BIOSYNTHESIS OF MEDIUM CHAIN LENGTH POLYHYDROXYALKANOATES

FIELD OF THE INVENTION

The invention relates to the biosynthesis of polymers and more specifically to the biosynthesis of polyhydroxyalkanoate polymers in plants. In particular, a transgenic plant producing peroxisome- or glyoxysome-targeted polyhydroxyalkanoate synthase resulting in the production of polyhydroxyalkanoate materials.

BACKGROUND OF THE INVENTION

PHAs are bacterial polyesters that accumulate in a wide variety of bacteria. These polymers have properties ranging from stiff and brittle plastics to rubber-like materials, and are biodegradable. Because of these properties, PHAs are an attractive source of nonpolluting plastics and elastomers.

Currently, there are approximately a dozen biodegradable plastics in commercial use that possess properties suitable for producing a number of specialty and commodity products (Lindsay, *Modern Plastics* 2: 62 (1992)). One such biodegradable plastic in the polyhydroxyalkanoate (PHA) family that is commercially important is Biopol™, a random copolymer of 3-hydroxybutyrate (3HB) and 3-hydroxyvalerate (3HV). This bioplastic is used to produce biodegradable molded material (e.g., bottles), films, coatings, and in drug release applications. Biopol™ is produced via a fermentation process employing the bacterium *Alcaligenes eutrophus* (Byrom, *Trends Biotechnol.* 5: 246 (1987)). The current market price is $6-7/lb, and the annual production is 1,000 tons. By best estimates, this price can be reduced only about 2-fold via fermentation (Poirier et al., *Bio/Technology* 13: 142 (1995)). Competitive synthetic plastics such as polypropylene and polyethylene cost about 35-45¢/lb (Layman, *Chem. & Eng. News*, p. 10 (Oct. 31, 1994). The annual global demand for polyethylene alone is about 37 million metric tons (Layman, *Chem. & Eng. News*, p. 10 (Oct. 31, 1994). It is therefore likely that the cost of producing P(3HB-co-3HV) by microbial fermentation will restrict its use to low-volume specialty applications.

Polyhydroxy oate (PHA) is a family of polymers mposed primarily of R-3-hydroxyalkanoic acids (Anderson, A. J. & Dawes, E. A. *Microbiol. Rev.* 54: 450-472. (1990); Steinbüchel, A. in *Novel Biomaterials from Biological Sources*, ed. Byrom, D. (MacMillan, New York), pp. 123-213. (1991); Poirier, Y. Nawrath, C. & Somerville, C. *Bio/Technology* 13: 143-150 (1995)). Polyhydroxybutyrate (PHB) is the most well characterized PHA. High molecular weight PHB is found as intracellular inclusions in a wide variety of bacteria (Steinbüchel, A. in *Novel Biomaterials from Biological Sources*, ed. Byrom, D. (MacMillan, New York), pp. 123-213. (1991)). In *Alcaligenes eutrophus*, PHB typically accumulates to 80% dry weight with inclusions being typically 0.2-1 μm in diameter. Small quantity of PHB oligomers of approximately 150 monomer units are also found associated with membranes of bacteria and eukaryotes, where they form channels permeable to calcium (Reusch, R. N., *Can. J. Microbiol.* 41 (Suppl. 1): 50-54 (1995)). High molecular weight PHAs have the properties of thermoplastics and elastomers. Numerous bacteria and fungi can hydrolyze PHAs to monomers and oligomers, which are metabolized as a carbon source. PHAs have, thus, attracted attention as a potential source of renewable and biodegradable plastics and elastomers. PHB is a highly crystalline polymer with rather poor physical properties, being relatively stiff and brittle (de Koning, G., *Can. J. Microbiol.* 41 (Suppl. 1): 303-309 (1995)). In contrast, PHA copolymers containing monomer units ranging from 3 to 5 carbons for short-chain-length PHA (SCL-PHA), or 6 to 14 carbons for medium-chain-length PHA (MCL-PHA), are less crystalline and more flexible polymers (de Koning, G., *Can. J. Microbiol.* 41 (Suppl. 1): 303-309 (1995)).

PHB has been produced in the plant *Arabidopsis thaliana* expressing the *A. eutrophus* PHB biosynthetic enzymes (Poirier, Y., et al., *Science* 256: 520-523 (1992); Nawrath, C., et al., *Proc. Natl. Acad. Sci. U.S.A.* 91: 12760-12764 (1994)). In plants expressing the PHB pathway in the plastids, leaves accumulated up to 14% PHB per gram dry weight (Nawrath, C., et al., *Proc. Natl. Acad. Sci. U.S.A.* 91: 12760-12764 (1994)). High-level synthesis of PHB in plants opened the possibility of utilizing agricultural crops as a suitable system for the production of PHAs on a large scale and at low cost (Poirier, Y. et al., *Bio/Technology* 13: 143-150 (1995); Poirier, Y., et al., *FEMS Microbiol. Rev.* 103: 237-246 (1992); Nawrath, C., et al. *Molecular Breeding* 1: 105-22 (1995)). PHB was also

shown to be synthesized in insect cells expressing a mutant fatty acid synthase (Williams, M. D., et al., *Appl. Environ. Microbiol.* 62: 2540-2546 (1996)), and in yeast expressing the *A. eutrophus* PHB synthase (Leaf, T. A., et al. *Microbiol.* 142: 1169-1180 (1996)).

A number of pseudomonads, including *Pseudomonas putida* and *Pseudomonas aeruginosa*, accumulate MCL-PHAs when cells are grown on alkanoic acids (Anderson, A. J. & Dawes, E. A. *Microbiol. Rev.* 54: 450-472. (1990); Steinbüchel, A. in *Novel Biomaterials from Biological Sources*, ed. Byrom, D. (MacMillan, New York), pp. 123-213. (1991); Poirier, Y. Nawrath, C. & Somerville, C. *Bio/Technology* 13: 143-150 (1995)). The nature of the PHA produced is related to the substrate used for growth and is typically composed of monomers which are 2n carbons shorter than the substrate. These studies indicate that MCL-PHAs are synthesized by the PHA synthase from 3-hydroxyacyl-CoA intermediates generated by the β-oxidation of alkanoic acids (Huijberts, G. N. M., et al. *Appl. Environ. Microbiol.* 58: 536-544 (1992); Huijberts, G. N. M., et al., *J. Bacteriol.* 176: 1661-1666 (1994)).

There exists a need for novel methods towards the biosynthesis of polyhydroxyalkanoate materials suitable for commercial applications. Towards this goal, this patent application discloses the materials and methods for the use of a peroxisome targeted polyhydroxyalkanoate synthase protein in the biosynthesis of polyhydroxyalkanoate polymers. Localization in the peroxisomes allow for the utilization of intermediates from the lipid β-oxidation pathway. Plants expressing a *P. aeruginosa* polyhydroxyalkanoate synthase modified for peroxisome targeting produce PHA containing saturated and unsaturated 3-hydroxyalkanoic acids ranging from 6 to 16 carbons. Polyhydroxyalkanoate granules are found within the glyoxysomes or leaf-type peroxisomes of dark-and light-grown plants, respectively, as well as in the vacuoles.

SUMMARY OF THE INVENTION

The invention is directed towards materials and methods for the biosynthesis of polyhydroxyalkanoate polymers. More particularly, a fusion protein comprising a

polyhydroxyalkanoate synthase protein subunit and a peroxisome targeting protein subunit renders a host cell or plant capable of producing polyhydroxyalkanoate polymer materials.

In one embodiment, the invention provides a non-naturally ocurring fusion protein comprising a peroxisome targeting protein subunit and a polyhydroxyalkanoate synthase protein subunit. Generally, the peroxisome targeting protein subunit and the polyhydroxyalkanoate synthase protein subunit may be any subunit suitable for participation in the invention. The peroxisome targeting subunit may be an N-terminal or C-terminal subunit. The N-terminal subunit is preferably PTS2. The C-terminal peroxisome targeting subunit preferably comprises a tripeptide. The first amino acid in the N-terminus to C-terminus direction is preferably S, A, or P. The second amino acid in the N-terminus to C-terminus direction is preferably K, R, S, or H. The third amino acid in the N-terminus to C-terminus direction is L, M, I, or F. More preferably, the C-terminal peroxisome targeting subunit comprises ARM, SRM, SKL, ARL, SRL, PSI, or PRM. The peroxisome targeting subunit is preferably at least 70% identical to SEQ ID NO:14, more preferably at least 80% identical to SEQ ID NO:14, even more preferably at least 90% identical to SEQ ID NO:14, and most preferably is SEQ ID NO:14. The polyhydroxyalkanoate synthase protein subunit is preferably a *Pseudomonas* subunit, and more preferably a *Pseudomonas aeruginosa* subunit. The polyhydroxyalkanoate synthase protein subunit may preferably be either a PHAC1 or PHAC2 subunit. The PHAC1 subunit is preferably at least 70% identical to SEQ ID NO:2, more preferably at least 80% identical to SEQ ID NO:2, even more preferably at least 90% identical to SEQ ID NO:2, and most preferably is SEQ ID NO:2. The PHAC2 subunit is preferably at least 70% identical to SEQ ID NO:4, more preferably at least 80% identical to SEQ ID NO:4, even more preferably at least 90% identical to SEQ ID NO:4, and most preferably is SEQ ID NO:4. The fusion protein is preferably at least 70% identical to SEQ ID NO:18 or SEQ ID NO:20, more preferably at least 80% identical to SEQ ID NO:18 or SEQ ID NO:20, even more preferably at least 90% identical to SEQ ID NO:18 or SEQ ID NO:20, and most preferably is SEQ ID NO:18 or SEQ ID NO:20.

In an alternative embodiment, the invention encompasses a nucleic acid segment encoding a non-naturally occurring fusion protein. The nucleic acid segment preferably comprises a nucleic acid sequence encoding a peroxisome targeting protein subunit, and a

nucleic acid sequence encoding a polyhydroxyalkanoate synthase protein subunit. The nucleic acid sequence encoding a peroxisome targeting protein subunit preferably comprises at least a 6 contiguous nucleic acid sequence from SEQ ID NO:13. The length of the contiguous nucleic acid sequence may be 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, etcetera, 50, 51, 52, etcetera, 100, 101, 102, etcetera, up to and including the entire length of SEQ ID NO:13. The nucleic acid sequence encoding a peroxisome targeting protein subunit is preferably at least 70% identical to SEQ ID NO:13, more preferably at least 80% identical to SEQ ID NO:13, even more preferably at least 90% identical to SEQ ID NO:13, and most preferably is SEQ ID NO:13. The nucleic acid sequence encoding a peroxisome targeting protein subunit preferably hybridizes to SEQ ID NO:13. The nucleic acid sequence encoding a polyhydroxyalkanoate synthase protein subunit preferably comprises at least a 6 contiguous nucleic acid sequence from SEQ ID NO:1, SEQ ID NO:3, SEQ ID NO:15, or SEQ ID NO:16. The length of the contiguous nucleic acid sequence may be 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, etcetera, 50, 51, 52, etcetera, 100, 101, 102, etcetera, up to and including the entire length of SEQ ID NO:1, SEQ ID NO:3, SEQ ID NO:15, or SEQ ID NO:16. The nucleic acid sequence encoding a polyhydroxyalkanoate synthase protein subunit is preferably at least 70% identical to SEQ ID NO:1, SEQ ID NO:3, SEQ ID NO:15, or SEQ ID NO:16, more preferably at least 80% identical to SEQ ID NO:1, SEQ ID NO:3, SEQ ID NO:15, or SEQ ID NO:16, even more preferably at least 90% identical to SEQ ID NO:1, SEQ ID NO:3, SEQ ID NO:15, or SEQ ID NO:16, further preferably is SEQ ID NO:1, SEQ ID NO:3, SEQ ID NO:15, or SEQ ID NO:16, and most preferably is SEQ ID NO:15 or SEQ ID NO:16. The nucleic acid sequence encoding a polyhydroxyalkanoate synthase protein subunit preferably hybridizes to SEQ ID NO:1, SEQ ID NO:3, SEQ ID NO:15, or SEQ ID NO:16. The encoded peroxisome targeting protein subunit may be an N-terminal or C-terminal peroxisome targeting protein subunit. The encoded N-terminal peroxisome targeting subunit is preferably PTS-2. The encoded C-terminal peroxisome targeting protein subunit preferably comprises a tripeptide. The tripeptide preferably comprises a first amino acid in the N-terminus to C-terminus direction being S, A, or P; a second amino acid in the N-terminus to C-terminus direction being K, R, S, or H; and a third amino acid in the N-terminus to C-terminus direction being L, M, I, or F. The encoded tripeptide preferably is ARM, SRM, SKL, ARL, SRL, PSI, or PRM. The nucleic acid

sequence encoding a polyhydroxyalkanoate synthase protein subunit preferably encodes at least a 5 contiguous amino acid sequence from SEQ ID NO:2 or SEQ ID NO:4. The length of the contiguous nucleic acid sequence may be 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, etcetera, 50, 51, 52, etcetera, 100, 101, 102, etcetera, up to and including the entire length of SEQ ID NO:2 or SEQ ID NO:4. The nucleic acid sequence encoding a polyhydroxyalkanoate synthase protein subunit preferably encodes an amino acid sequence at least 70% identical to SEQ ID NO:2 or SEQ ID NO:4, more preferably at least 80% identical to SEQ ID NO:2 or SEQ ID NO:4, even more preferably at least 90% identical to SEQ ID NO:2 or SEQ ID NO:4, and most preferably is SEQ ID NO:2 or SEQ ID NO:4.

In an alternative embodiment, the invention discloses a recombinant vector comprising in the 5' to 3' direction a) a promoter that directs transcription of a structural nucleic acid sequence encoding a non-naturally occurring fusion protein, wherein the fusion protein comprises a peroxisome targeting protein subunit and a polyhydroxyalkanoate synthase protein subunit, b) a structural nucleic acid sequence encoding a non-naturally occurring fusion protein, wherein the fusion protein comprises a peroxisome targeting protein subunit and a polyhydroxyalkanoate synthase protein subunit, and c) a 3' transcription terminator. The recombinant vector may further comprise a 3' polyadenylation signal sequence that directs the addition of polyadenylate nucleotides to the 3' end of RNA transcribed from the structural nucleic acid coding sequence. The recombinant vector may further comprise a selectable marker. The selectable marker may generally be any selectable marker suitable for the intended host cell or plant, and preferably is a kanamycin resistance marker, a hygromycin resistance marker, or a herbicide resistance marker. The promoter may be constitutive, inducible, tissue specific, or combinations thereof. The constitutive promoter may generally any constitutive promoter suitable for the intended host cell or plant, and preferably is CaMV35S, enhanced CaMV35S, FMV, mas, nos, or ocs. The inducible promoter may generally be any inducible promoter suitable for the intended host cell or plant, and preferably is tac, salicylic acid induced, polyacrylic acid induced, safener induced, heat shock promoter, nitrate induced, hormone induced, or light induced. The tissue specific promoter may generally be any tissue specific promoter suitable for the intended host cell or plant, and preferably is the β-conglycinin 7S promoter,

napin promoter, phaseolin promoter, zein promoter, soybean trypsin inhibitor promoter, ACP promoter, stearoyl-ACP desaturase promoter, or oleosin promoter. The nucleic acid sequence encoding a peroxisome targeting protein subunit preferably comprises at least a 6 contiguous nucleic acid sequence from SEQ ID NO:13. The length of the contiguous nucleic acid sequence may be 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, etcetera, 50, 51, 52, etcetera, 100, 101, 102, etcetera, up to and including the entire length of SEQ ID NO:13. The nucleic acid sequence encoding a peroxisome targeting protein subunit is preferably at least 70% identical to SEQ ID NO:13, more preferably at least 80% identical to SEQ ID NO:13, even more preferably at least 90% identical to SEQ ID NO:13, and most preferably is SEQ ID NO:13. The nucleic acid sequence encoding a peroxisome targeting protein subunit preferably hybridizes to SEQ ID NO:13. The encoded peroxisome targeting protein subunit may be an N-terminal or C-terminal peroxisome targeting protein subunit. The encoded N-terminal peroxisome targeting subunit is preferably PTS-2. The encoded C-terminal peroxisome targeting protein subunit preferably comprises a tripeptide. The tripeptide preferably comprises a first amino acid in the N-terminus to C-terminus direction being S, A, or P; a second amino acid in the N-terminus to C-terminus direction being K, R, S, or H; and a third amino acid in the N-terminus to C-terminus direction being L, M, I, or F. The encoded tripeptide preferably is ARM, SRM, SKL, ARL, SRL, PSI, or PRM. The encoded polyhydroxyalkanoate synthase protein subunit is preferably a *Pseudomonas* subunit, and more preferably is a *Pseudomonas aeruginosa* subunit. The nucleic acid sequence encoding a polyhydroxyalkanoate synthase protein subunit preferably comprises at least a 6 contiguous nucleic acid sequence from SEQ ID NO:1, SEQ ID NO:3, SEQ ID NO:15, or SEQ ID NO:16. The length of the contiguous nucleic acid sequence may be 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, etcetera, 50, 51, 52, etcetera, 100, 101, 102, etcetera, up to and including the entire length of SEQ ID NO:1, SEQ ID NO:3, SEQ ID NO:15, or SEQ ID NO:16. The nucleic acid sequence encoding a polyhydroxyalkanoate synthase protein subunit is preferably at least 70% identical to SEQ ID NO:1, SEQ ID NO:3, SEQ ID NO:15, or SEQ ID NO:16, more preferably at least 80% identical to SEQ ID NO:1, SEQ ID NO:3, SEQ ID NO:15, or SEQ ID NO:16, even more preferably at least 90% identical to SEQ ID NO:1, SEQ ID NO:3, SEQ ID NO:15, or SEQ ID NO:16, further preferably is SEQ ID NO:1, SEQ ID NO:3, SEQ ID NO:15, or SEQ ID NO:16, and most

preferably is SEQ ID NO:15 or SEQ ID NO:16. The nucleic acid sequence encoding a polyhydroxyalkanoate synthase protein subunit preferably hybridizes to SEQ ID NO:1, SEQ ID NO:3, SEQ ID NO:15, or SEQ ID NO:16. The nucleic acid sequence encoding a polyhydroxyalkanoate synthase protein subunit preferably encodes at least a 5 contiguous amino acid sequence from SEQ ID NO:2 or SEQ ID NO:4. The length of the contiguous nucleic acid sequence may be 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, etcetera, 50, 51, 52, etcetera, 100, 101, 102, etcetera, up to and including the entire length of SEQ ID NO:2 or SEQ ID NO:4. The nucleic acid sequence encoding a polyhydroxyalkanoate synthase protein subunit preferably encodes an amino acid sequence at least 70% identical to SEQ ID NO:2 or SEQ ID NO:4, more preferably at least 80% identical to SEQ ID NO:2 or SEQ ID NO:4, even more preferably at least 90% identical to SEQ ID NO:2 or SEQ ID NO:4, and most preferably is SEQ ID NO:2 or SEQ ID NO:4. The structural nucleic acid sequence preferably comprises SEQ ID NO:17 or SEQ ID NO:19, and preferably encodes SEQ ID NO:18 or SEQ ID NO:20.

In an alternative embodiment, the invention encompasses a recombinant host cell comprising a nucleic acid segment encoding a non-naturally occurring fusion protein, wherein the nucleic acid segment comprises a nucleic acid sequence encoding a peroxisome targeting protein subunit and a nucleic acid sequence encoding a polyhydroxyalkanoate synthase protein subunit. The recombinant host cell may generally be any type of host cell, and preferably is a fungal or plant host cell. The fungal cell is generally any type of fungal cell, and preferably a *Schizosaccharomyces pombe*, *Streptomyces rimofaciens*, *Fusarium*, *Aspergillus niger*, or *Saccharomyces cerevisiae* cell. The plant cell is generally any type of plant cell, and preferably an alfalfa, banana, barley, bean, cabbage, canola/oilseed rape, carrot, castorbean, celery, clover, coconut, corn, cotton, cucumber, linseed, melon, olive, palm, parsnip, pea, peanut, pepper, potato, potato, radish, rapeseed, rice, soybean, spinach, sunflower, tobacco, tomato, or wheat cell. The recombinant host cell may further comprise a nucleic acid segment encoding an acyl-ACP thioesterase, a fatty acyl hydroxylase, a yeast multifunctional protein (MFP), or an hydroxyacyl-CoA epimerase.

A further alternative embodiment describes a genetically transformed plant cell comprising in the 5' to 3' direction: a) a promoter to direct transcription of a structural nucleic acid sequence encoding a non-naturally occurring fusion protein, wherein the structural nucleic acid sequence comprises: i) a nucleic acid sequence encoding a peroxisome targeting protein subunit; and ii) a nucleic acid sequence encoding a polyhydroxyalkanoate synthase protein subunit; b) a structural nucleic acid sequence encoding a non-naturally occurring fusion protein, wherein the structural nucleic acid sequence comprises: i) a nucleic acid sequence encoding a peroxisome targeting protein subunit; and ii) a nucleic acid sequence encoding a polyhydroxyalkanoate synthase protein subunit; c) a 3' transcription terminator sequence; and d) a 3' polyadenylation signal sequence that directs the addition of polyadenylate nucleotides to the 3' end of RNA transcribed from the structural nucleic acid coding sequence. The plant cell is generally any type of plant cell, and preferably an alfalfa, banana, barley, bean, cabbage, canola/oilseed rape, carrot, castorbean, celery, clover, coconut, corn, cotton, cucumber, linseed, melon, olive, palm, parsnip, pea, peanut, pepper, potato, potato, radish, rapeseed, rice, soybean, spinach, sunflower, tobacco, tomato, or wheat cell. The plant cell may further comprise a nucleic acid segment encoding an acyl-ACP thioesterase, a fatty acyl hydroxylase, a yeast multifunctional protein (MFP), or an hydroxyacyl-CoA epimerase.

An additional embodiment describes a genetically transformed plant comprising in the 5' to 3' direction: a) a promoter to direct transcription of a structural nucleic acid sequence encoding a non-naturally occurring fusion protein, wherein the structural nucleic acid sequence comprises: i) a nucleic acid sequence encoding a peroxisome targeting protein subunit; and ii) a nucleic acid sequence encoding a polyhydroxyalkanoate synthase protein subunit; b) a structural nucleic acid sequence encoding a non-naturally occurring fusion protein, wherein the structural nucleic acid sequence comprises: i) a nucleic acid sequence encoding a peroxisome targeting protein subunit; and ii) a nucleic acid sequence encoding a polyhydroxyalkanoate synthase protein subunit; c) a 3' transcription terminator sequence; and d) a 3' polyadenylation signal sequence that directs the addition of polyadenylate nucleotides to the 3' end of RNA transcribed from the structural nucleic acid coding sequence. The plant may generally be any type of plant, and preferably an alfalfa, banana,

barley, bean, cabbage, canola/oilseed rape, carrot, castorbean, celery, clover, coconut, corn, cotton, cucumber, linseed, melon, olive, palm, parsnip, pea, peanut, pepper, potato, potato, radish, rapeseed, rice, soybean, spinach, sunflower, tobacco, tomato, or wheat plant. The promoter may be constitutive, inducible, tissue specific, or combinations thereof. The constitutive promoter may generally any constitutive promoter suitable for the intended plant, and preferably is CaMV35S, enhanced CaMV35S, FMV, mas, nos, or ocs. The inducible promoter may generally be any inducible promoter suitable for the intended plant, and preferably is tac, salicylic acid induced, polyacrylic acid induced, safener induced, heat shock promoter, nitrate induced, hormone induced, or light induced. The tissue specific promoter is generally any tissue specific promoter, and preferably is the β-conglycinin 7S promoter, napin promoter, phaseolin promoter, zein promoter, soybean trypsin inhibitor promoter, ACP promoter, stearoyl-ACP desaturase promoter, or oleosin promoter. The plant may further comprise a nucleic acid segment encoding an acyl-ACP thioesterase, a fatty acyl hydroxylase, a yeast multifunctional protein (MFP), or an hydroxyacyl-CoA epimerase.

The invention describes a method for preparing host cells useful to produce a non-naturally occurring fusion protein comprising the steps of: a) selecting a host cell b) transforming the selected host cell with a recombinant vector having a structural nucleic acid sequence encoding a non-naturally occurring fusion protein, wherein the structural nucleic acid sequence comprises: i) a nucleic acid sequence encoding a peroxisome targeting protein subunit; and ii) a nucleic acid sequence encoding a polyhydroxyalkanoate synthase protein subunit; and c) obtaining transformed host cells. The vector may further comprise a selectable marker. The selectable marker may generally be any selectable marker suitable for use in the intended host cell, and more preferably for plants is a kanamycin resistance marker, a hygromycin resistance marker, or a herbicide resistance marker. The host cell may generally be any type of cell, and preferably is a fungal or plant cell. The fungal cell may generally be any type of fungal cell, and more preferably is a *Schizosaccharomyces pombe*, *Streptomyces rimofaciens*, *Fusarium*, *Aspergillus niger*, or *Saccharomyces cerevisiae* cell. The plant cell may generally be any type of plant cell, and more preferably is an alfalfa, banana, barley, bean, cabbage, canola/oilseed rape, carrot,

castorbean, celery, clover, coconut, corn, cotton, cucumber, linseed, melon, olive, palm, parsnip, pea, peanut, pepper, potato, potato, radish, rapeseed, rice, soybean, spinach, sunflower, tobacco, tomato, or wheat cell.

The invention further describes a method of preparing a transformed plant useful to produce a non-naturally occurring fusion protein comprising the steps of: a) selecting a host plant cell b) transforming the selected host cell with a recombinant vector having a structural nucleic acid sequence encoding a non-naturally occurring fusion protein, wherein the structural nucleic acid sequence comprises: i) a nucleic acid sequence encoding a peroxisome targeting protein subunit; and ii) a nucleic acid sequence encoding a polyhydroxyalkanoate synthase protein subunit; c) obtaining transformed host plant cells; and d) regenerating the transformed host plant cells. The vector may further comprise a selectable marker. The selectable marker may generally be any selectable marker suitable for use in the intended host cell, and more preferably is a kanamycin resistance marker, a hygromycin resistance marker, or a herbicide resistance marker. The host plant cell may generally be any type of plant cell, and more preferably is an alfalfa, banana, barley, bean, cabbage, canola/oilseed rape, carrot, castorbean, celery, clover, coconut, corn, cotton, cucumber, linseed, melon, olive, palm, parsnip, pea, peanut, pepper, potato, potato, radish, rapeseed, rice, soybean, spinach, sunflower, tobacco, tomato, or wheat cell. The invention also encompasses the plant made by the above described methods.

A preferred embodiment is a method for the preparation of a polyhydroxyalkanoate, comprising the steps of: a) obtaining a cell capable of producing a non-naturally occurring fusion protein, wherein the fusion protein comprises: i) a peroxisome targeting protein subunit; and ii) a polyhydroxyalkanoate synthase protein subunit; b) establishing a culture of the cell; and c) culturing the cell under conditions suitable for the production of the polyester. The method may further comprise isolating the polyhydroxyalkanoate from the cultured cell. The culture may further comprise fatty acids, and more preferably natural fatty acids, non-natural or synthetic fatty acids, or mixtures thereof. The cell may generally be any type of cell, and preferably is a fungal or plant cell. The fungal cell may generally be any type of fungal cell, and more preferably is a *Schizosaccharomyces pombe*, *Streptomyces*

rimofaciens, *Fusar[redacted]*, *Aspergillus niger*, or *Saccharomyces [redacted]visiae* cell. The plant cell may generally be any type of plant cell, and more preferably is an alfalfa, banana, barley, bean, cabbage, canola/oilseed rape, carrot, castorbean, celery, clover, coconut, corn, cotton, cucumber, linseed, melon, olive, palm, parsnip, pea, peanut, pepper, potato, potato, radish, rapeseed, rice, soybean, spinach, sunflower, tobacco, tomato, or wheat cell. The polyhydroxyalkanoate isolated from the cell may generally be any type of polyhydroxyalkanoate, and preferably comprises 3-hydroxyhexanoic acid (H:6), 3-hydroxyoctanoic acid (H:8), 3-hydroxydecanoic acid (H:10), 3-hydroxydodecanoic acid (H:12), 3-hydroxytetradecanoic acid (H:14), 3-hydroxyhexadecanoic acid (H:16), 3-hydroxyheptanoic acid (H:7), 3-hydroxynonanoic acid (H9), 3-hydroxyundecanoic acid (H:11), 3-hydroxytridecanoic acid (H:13), 3-hydroxyhexadecatrienoic acid (H16:3), 3-hydroxyhexadecadienoic acid (H16:2), 3-hydroxyhexadecenoic acid (H16:1), 3-hydroxytetradecatrienoic acid (H14:3), 3-hydroxytetradecadienoic acid (H14:2), 3-hydroxytetradecenoic acid (H14:1), 3-hydroxydodecadienoic acid (H12:2), 3-hydroxydodecenoic acid (H12:1), 3-hydroxyoctenoic acid (H8:1), 4-hydroxydecanoic acid, 8-methyl-3-hydroxynonanoic acid, or 6-methyl-3-hydroxyheptanoic acid monomers.

In a further preferred embodiment, the invention presents a method for the preparation of a polyhydroxyalkanoate, comprising the steps of: a) obtaining a plant capable of producing a non-naturally occurring fusion protein, wherein the fusion protein comprises: i) a peroxisome targeting protein subunit; and ii) a polyhydroxyalkanoate synthase protein subunit; and c) growing the plant under conditions suitable for the production of the polyhydroxyalkanoate. The method may further comprise the step of isolating the polyhydroxyalkanoate from the plant. The method may further comprise supplementing the plant with natural fatty acids, non-natural fatty acids, or mixtures thereof. The plant may generally be any type of plant, and preferably is an alfalfa, banana, barley, bean, cabbage, canola/oilseed rape, carrot, castorbean, celery, clover, coconut, corn, cotton, cucumber, linseed, melon, olive, palm, parsnip, pea, peanut, pepper, potato, potato, radish, rapeseed, rice, soybean, spinach, sunflower, tobacco, tomato, or wheat plant. The polyhydroxyalkanoate isolated from the plant may generally be any type of polyhydroxyalkanoate, and preferably comprises 3-hydroxyhexanoic acid (H:6), 3-

hydroxyoctanoic acid (H:8), 3-hydroxydecanoic acid (H:10), 3-hydroxydodecanoic acid (H:12), 3-hydroxytetradecanoic acid (H:14), 3-hydroxyhexadecanoic acid (H:16), 3-hydroxyheptanoic acid (H:7), 3-hydroxynonanoic acid (H9), 3-hydroxyundecanoic acid (H:11), 3-hydroxytridecanoic acid (H:13), 3-hydroxyhexadecatrienoic acid (H16:3), 3-hydroxyhexadecadienoic acid (H16:2), 3-hydroxyhexadecenoic acid (H16:1), 3-hydroxytetradecatrienoic acid (H14:3), 3-hydroxytetradecadienoic acid (H14:2), 3-hydroxytetradecenoic acid (H14:1), 3-hydroxydodecadienoic acid (H12:2), 3-hydroxydodecenoic acid (H12:1), 3-hydroxyoctenoic acid (H8:1), 4-hydroxydecanoic acid, 8-methyl-3-hydroxynonanoic acid, or 6-methyl-3-hydroxyheptanoic acid monomers.

The invention further encompasses plants containing polyhydroxyalkanoates, wherein the polyhydroxyalkanoate comprises 3-hydroxyhexanoic acid (H:6), 3-hydroxyoctanoic acid (H:8), 3-hydroxydecanoic acid (H:10), 3-hydroxydodecanoic acid (H:12), 3-hydroxytetradecanoic acid (H:14), 3-hydroxyhexadecanoic acid (H:16), 3-hydroxyheptanoic acid (H:7), 3-hydroxynonanoic acid (H9), 3-hydroxyundecanoic acid (H:11), 3-hydroxytridecanoic acid (H:13), 3-hydroxyhexadecatrienoic acid (H16:3), 3-hydroxyhexadecadienoic acid (H16:2), 3-hydroxyhexadecenoic acid (H16:1), 3-hydroxytetradecatrienoic acid (H14:3), 3-hydroxytetradecadienoic acid (H14:2), 3-hydroxytetradecenoic acid (H14:1), 3-hydroxydodecadienoic acid (H12:2), 3-hydroxydodecenoic acid (H12:1), 3-hydroxyoctenoic acid (H8:1), 4-hydroxydecanoic acid, 8-methyl-3-hydroxynonanoic acid, or 6-methyl-3-hydroxyheptanoic acid monomers.

In an alternative embodiment, the invention describes polyhydroxyalkanoates comprising 3-hydroxyhexadecatrienoic acid (H16:3), 3-hydroxyhexadecadienoic acid (H16:2), 3-hydroxytetradecatrienoic acid (H14:3), or 3-hydroxydodecadienoic acid (H12:2) monomers.

DESCRIPTION OF THE FIGURES

The following figure forms part of the present specification and is included to further demonstrate certain aspects of the present invention. The invention may be better

understood by reference to the figure in combination with the detailed description of specific embodiments presented herein.

Figure 1: GC-MS analysis of PHA in transgenic plants. Trans-esterified chloroform extracts from phaC1-transformed line 3.3 (A, B) and vector-transformed line 21 (C, D) were analyzed. In panels A and C, the total ion chromatogram is presented, while on panel B and D, only ions with a mass-to-charge ratio of 103 are shown.

DESCRIPTION OF THE SEQUENCE LISTINGS

The following sequence listings form part of the present specification and are included to further demonstrate certain aspects of the present invention. The invention may be better understood by reference to one or more of these sequence listings in combination with the detailed description of specific embodiments presented herein.

SEQ ID NO:1	Wild type PHA synthase C1 nucleic acid sequence.
SEQ ID NO:2	Wild type PHA synthase C1 protein sequence.
SEQ ID NO:3	Wild type PHA synthase C2 nucleic acid sequence.
SEQ ID NO:4	Wild type PHA synthase C2 protein sequence.
SEQ ID NO:5	Forward PCR primer for PHA synthase C1 fusion sequence.
SEQ ID NO:6	Reverse PCR primer for PHA synthase C1 fusion sequence.
SEQ ID NO:7	Forward PCR primer for PHA synthase C2 fusion sequence.
SEQ ID NO:8	Reverse PCR primer for PHA synthase C2 fusion sequence.
SEQ ID NO:9	Wild type isocitrate lyase nucleic acid sequence.
SEQ ID NO:10	Wild type isocitrate lyase protein sequence.
SEQ ID NO:11	Forward PCR primer for isocitrate lyase fusion sequence.
SEQ ID NO:12	Reverse PCR primer for isocitrate lyase fusion sequence.
SEQ ID NO:13	Nucleic acid sequence encoding the isocitrate lyase peroxisome targeting protein subunit.
SEQ ID NO:14	Isocitrate lyase peroxisome targeting protein subunit.
SEQ ID NO:15	PHA synthase C1 nucleic acid sequence with plant preferred codon.

SEQ ID NO:16	PHA synthase C2 nucleic acid sequence with plant preferred codon.
SEQ ID NO:17	Nucleic acid sequence encoding PHA synthase C1 and isocitrate lyase fusion protein.
SEQ ID NO:18	PHA synthase C1 and isocitrate lyase fusion protein.
SEQ ID NO:19	Nucleic acid sequence encoding PHA synthase C2 and isocitrate lyase fusion protein.
SEQ ID NO:20	PHA synthase C2 and isocitrate lyase fusion protein.
SEQ ID NO:21	PCR amplified nucleic acid sequence encoding wild type *Candida albicans* MFP.
SEQ ID NO:22	Wild type *Candida albicans* MFP protein.
SEQ ID NO:23	PCR amplified nucleic acid sequence encoding SKL mutant *Candida albicans* MFP.
SEQ ID NO:24	*Candida albicans* MFP protein with SKL substitution for AKI.
SEQ ID NO:25	PCR amplified nucleic acid sequence encoding mutant *Candida albicans* MFP lacking AKI sequence.
SEQ ID NO:26	*Candida albicans* MFP protein lacking AKI sequence.

DEFINITIONS

The following definitions are provided in order to aid those skilled in the art in understanding the detailed description of the present invention.

"Acyl-ACP thioesterase" refers to proteins which catalyze the hydrolysis of acyl-ACP thioesters.

"C-terminal region" refers to the region of a peptide, polypeptide, or protein chain from the middle thereof to the end that carries the amino acid having a free a carboxyl group (the C-terminus).

"CoA" refers to coenzyme A.

The phrases "coding sequence", "open reading frame", and "structural sequence" refer to the region of continuous sequential nucleic acid triplets encoding a protein, polypeptide, or peptide sequence.

The term "encoding DNA" or "encoding nucleic acid" refers to chromosomal nucleic acid, plasmid nucleic acid, cDNA, or synthetic nucleic acid which codes on expression for any of the proteins or fusion proteins discussed herein.

"Fatty acyl hydroxylase" refers to proteins which catalyze the conversion of fatty acids to hydroxylated fatty acids.

The term "gene" refers to chromosomal DNA, plasmid DNA, cDNA, synthetic DNA, or other DNA that encodes a peptide, polypeptide, protein, or RNA molecule, and regions flanking the coding sequence involved in the regulation of expression.

The term "genome" as it applies to bacteria encompasses both the chromosome and plasmids within a bacterial host cell. Encoding DNAs of the present invention introduced into bacterial host cells can therefore be either chromosomally-integrated or plasmid-localized. The term "genome" as it applies to plant cells encompasses not only chromosomal DNA found within the nucleus, but organelle DNA found within subcellular components of the cell. DNAs of the present invention introduced into plant cells can therefore be either chromosomally-integrated or organelle-localized.

"Glyoxysome" and "peroxisome" refer to the same organelle in a plant. Glyoxysome refers to a type of peroxisome found in germinating seedlings, senescing tissues, or in dark-grown tissues. Glyoxysomes and peroxisomes contain enzymes responsible for the conversion of lipids to carbohydrates.

"Identity" refers to the degree of similarity between two nucleic acid or protein sequences. An alignment of the two sequences is performed by a suitable computer

program. A widely used and accepted computer program for performing sequence alignments is CLUSTALW v1.6 (Thompson, et al. *Nucl. Acids Res.*, 22: 4673-4680 (1994)). The number of matching bases or amino acids is divided by the total number of bases or amino acids, and multiplied by 100 to obtain a percent identity. For example, if two 580 base pair sequences had 145 matched bases, they would be 25 percent identical. If the two compared sequences are of different lengths, the number of matches is divided by the shorter of the two lengths. For example, if there were 100 matched amino acids between 200 and a 400 amino acid proteins, they are 50 percent identical with respect to the shorter sequence.

The terms "microbe" or "microorganism" refer to algae, bacteria, fungi, and protozoa.

"N-terminal region" refers to the region of a peptide, polypeptide, or protein chain from the amino acid having a free a amino group to the middle of the chain.

"Nucleic acid" refers to ribonucleic acid (RNA) and deoxyribonucleic acid (DNA).

A "nucleic acid segment" is a nucleic acid molecule that has been isolated free of total genomic DNA of a particular species, or that has been synthesized. Included with the term "nucleic acid segment" are DNA segments, recombinant vectors, plasmids, cosmids, phagemids, phage, viruses, etcetera.

"Overexpression" refers to the expression of a polypeptide or protein encoded by a DNA introduced into a host cell, wherein said polypeptide or protein is either not normally present in the host cell, or wherein said polypeptide or protein is present in said host cell at a higher level than that normally expressed from the endogenous gene encoding said polypeptide or protein.

The term "plastid" refers to the class of plant cell organelles that includes amyloplasts, chloroplasts, chromoplasts, elaioplasts, eoplasts, etioplasts, leucoplasts, and proplastids. These organelles are self-replicating, and contain what is commonly referred to

as the "chloroplast genome," a circular DNA molecule that ranges in size from about 120 to about 217 kb, depending upon the plant species, and which usually contains an inverted repeat region (Fosket, Plant growth and Development, Academic Press, Inc., San Diego, CA, p. 132 (1994)).

"Polyadenylation signal" or "polyA signal" refers to a nucleic acid sequence located 3' to a coding region that directs the addition of adenylate nucleotides to the 3' end of the mRNA transcribed from the coding region.

The term "polyhydroxyalkanoate (or PHA) synthase" refers to enzymes that convert hydroxyacyl-CoAs to polyhydroxyalkanoates and free CoA.

The term "promoter" or "promoter region" refers to a nucleic acid sequence, usually found upstream (5') to a coding sequence, that controls expression of the coding sequence by controlling production of messenger RNA (mRNA) by providing the recognition site for RNA polymerase and/or other factors necessary for start of transcription at the correct site. As contemplated herein, a promoter or promoter region includes variations of promoters derived by means of ligation to various regulatory sequences, random or controlled mutagenesis, and addition or duplication of enhancer sequences. The promoter region disclosed herein, and biologically functional equivalents thereof, are responsible for driving the transcription of coding sequences under their control when introduced into a host as part of a suitable recombinant vector, as demonstrated by its ability to produce mRNA.

"Protein subunit" refers to a protein sequence that is part of a fusion protein. Examples are β-galactosidase, FLAG, green fluorescent protein, and in the instant invention, polyhydroxyalkanoate synthase, and a peroxisome or glyoxysome targetting peptide.

"PTS2" refers to an N-terminal protein subunit having the sequence (R/K)(L/Q/I)XXXXX(H/Q)L, wherein X is any amino acid.

"Regeneration" refers to the process of growing a plant from a plant cell (e.g., plant protoplast or explant).

"Transformation" refers to a process of introducing an exogenous nucleic acid sequence (e.g., a vector, recombinant nucleic acid molecule) into a cell or protoplast in which that exogenous nucleic acid is incorporated into a chromosome or is capable of autonomous replication.

A "transformed cell" or "transgenic cell" is a cell whose DNA has been altered by the introduction of an exogenous nucleic acid molecule into that cell.

A "transformed plant" or "transgenic plant" is a plant whose DNA has been altered by the introduction of an exogenous nucleic acid molecule into that plant, or by the introduction of an exogenous nucleic acid molecule into a plant cell from which the plant was regenerated or derived.

The following examples are included to demonstrate preferred embodiments of the invention. It should be appreciated by those of skill in the art that the techniques disclosed in the examples which follow represent techniques discovered by the inventors to function well in the practice of the invention, and thus can be considered to constitute preferred modes for its practice. However, those of skill in the art should, in light of the present disclosure, appreciate that many changes can be made in the specific embodiments which are disclosed and still obtain a like or similar result without departing from the spirit and scope of the invention.

EXAMPLES

EXAMPLE 1: Plant material

Arabidopsis thaliana, race Columbia, was transformed by the vacuum infiltration method (Bechtold, N., et al., *C.R. Acad. Sci. Paris* 316: 1194-1199 (1993)). Transformants

were selected on media containing Murashige and Skoog salts ("MS", Murashige, T. and Skoog, F., *Physiol. Plant.* 15: 473-497 (1962)), 1% (w/v) sucrose, 0.7% (w/v) agar and 50 µg/mL kanamycin. Kanamycin-resistant plants were subsequently transferred to soil and grown under continuous fluorescent light at 19°C. In some experiments, plants were grown under constant agitation (100 rpm) for 1-2 weeks in liquid media containing MS salts and 2% sucrose.

EXAMPLE 2: Cloning of peroxisomally targeted PHA synthases C1 and C2

The *phaC1* and *phaC2* genes were obtained from Steinbüchel (Timm, A. and Steinbüchel, A., *Eur. J. Biochem.* 209: 14-30 (1992), GenBank Accession Number X66592). PCR was used to amplify the genes and to modify their 5'- and 3'-termini as follows: At the 5'-end the codons encoding the serine-2 and the arginine-2 residue of *phaC1* and *phaC2*, respectively, were modified to conform more closely with the general codon preferences of *A. thaliana* (Meyerowitz, E. M. in *Methods in Arabidopsis research* , eds. Koncz, C., Chua, N.-H. & Schell, J. (World Scientific Publishing, Singapore), pp. 100-119 (1992)). At the 3'-end the sequences were modified to obtain suitable cloning sites and to delete the stop codons to enable the construction of chimerical fusions with the peroxisomal targeting sequence.

The carboxy-terminal 35 amino acid residues of the isocitrate lyase gene (ICL) (Olsen, L.J., et al., *Plant Cell* 5: 941-952 (1993), GenBank Accession Number Y13356) from *Brassica napus* were used as targeting sequence for the PHA synthases C1 and C2. It has been shown previously that this sequence was sufficient to ensure the peroxisomal localization of the chloramphenicol acetyl transferase (CAT) to the peroxisomes in *A. thaliana* (Comai, L. et al., *The Plant Cell* 1: 293-300 (1989); Olsen, L. J. et al., *The Plant Cell* 5: 941-952 (1993); Zhang, J. Z. et al., *Mol. Gen. Genet.* 238: 177-184 (1993)). A PCR product encoding the ICL targeting sequence was cloned into the vector pART7 (Gleaves, A.P., *Plant Mol. Biol.* 20: 1202-1207 (1992), GenBank Accession Number X69707). The PCR products containing the *phaC1* or *phaC2* genes were cloned 5'-upstream of the ICL sequence to produce a contiguous open reading frame encoding the targeted fusion proteins.

The 5'- and 3'-ends of the genes in the resulting plasmids pART7_phaC1_ICL and pART7_phaC2_ICL were sequenced to verify the modifications.

The PHA accumulation-deficient mutant *Pseudomonas putida* KT2440 NK2:3 was obtained from Steinbüchel for complementation studies to verify the enzyme activities of the modified PHA synthases C1 and C2. The *phaC1_ICL* and *phaC2_ICL* genes were cloned into the broad-host range plasmid pVLT35 behind the IPTG-inducible tac-promoter (Lorenzo, V. et al., *Gene* 123: 17-24 (1993)) and electroporated into the *P. putida* mutant. Streptomycin-resistant transformants were subcultured onto minimal medium containing either octanoate or gluconate as sole carbon source. The Nile Blue A fluorescence stain (Page, W. J. and C. J. Tenove, *Biotechnology Techniques* 10: 215-220 (1996)) was used to visualize PHA accumulation. Upon IPTG induction PHA accumulation was observed with pVLT35_phaC1_ICL and pVLT35_phaC2_ICL, but not with pVLT35 alone, thus indicating that the modified genes were still active.

EXAMPLE 3: Plant transformation and screening for PHA synthase C1 transgenic plants

The NotI-cassettes of plasmids pART7_phaC1_ICL and pART7_phaC2_ICL containing the modified genes flanked by the Cauliflower mosaic virus 35S promoter (CaMV35S) and the octapine synthase (ocs) 3'-terminator were cloned into the plant binary vector pART27 to obtain pART27_phaC1_ICL and pART27_phaC2_ICL. These plasmids were transformed into *A. thaliana* ecotype Columbia by *Agrobacterium* GV3101-mediated transfer utilizing an *in planta* vacuum-infiltration method (Bechtold, N. et al., *C.R. Acad. Sci. Paris* 316: 1194-1199 (1993)). Transgenic T1 plants were selected for antibiotic resistance during germination of the seeds of infiltrated plants on plant growth medium containing mineral salts, sucrose and kanamycin. Negative control plants containing only the insert-less T-DNA of the vector pART27 were obtained in the same way.

Transgenic PHAC1 plants (T1) expressing high amounts of PHA synthase C1 were selected by Western analysis with an antiserum against the PHA synthase C1, which was

obtained from Steinbüchel's laboratory. Unfortunately no antibodies against PHA synthase C2 were found to be suitable, so a different screening strategy was used, see below. Six independent lines expressing varying quantities of PHA synthase C1 were obtained from 12 originally infiltrated plants, which had been harvested individually (another 19 have not yet been investigated). Initially some problems with the western analysis were encountered, one of which was the precipitation of the PHA synthase in plant protein extracts upon freezing. Analysis of the kanamycin segregation of the second generation (T2) and third generation (T3) plants indicated that three of these lines contained multilocus T-DNA inserts. Initially these lines exhibited the highest expression of PHA synthase C1 as judged by western analysis, however, the expression of the transgene in these lines was variable in plants of the T2 and T3 generation and complete "silencing" was observed. The line PHAC1#3.3 was finally chosen for further studies, because it contained a single-locus T-DNA insert and exhibited stable expression of the transgene as seen on the western blot.

EXAMPLE 4: PHA production by PHAC1 plants

A protocol for the detection of monomers of PHA by gas chromatography was developed based on the method described for the extraction of PHB from Arabidopsis (Poirier, Y. et al., *Int. J. Biol. Macromol.* 17: 7-12 (1995)). Whole leaves were extracted several times with ethanol and methanol to elute all the soluble lipids, thereafter chloroform and methanol acidified with 3% (v/v) H_2SO_4 were added in equal volumes and the reactions were put at 98°C for 4 hours to transesterify the PHA polyester. GC-chromatograms of the resulting chloroform extracts showed a large number of peaks, most of which were due to the derivatization of various leave compounds. Peaks corresponding to the standards of the expected methyl esters of PHA monomers were, however, distinguishable amongst the others. A large fraction of the plant material was solubilized during this transesterification treatment, it was however not determined whether underivatized PHA remained in the solid underivatized material. This made the quantification of the PHA in plant material slightly uncertain, but the authors estimated intuitively that most of the PHA in the material became derivatized preferentially. The GC-standards (from Sigma Chemical, St. Louis, MO, except H6 which was from Beat Keller) were the methyl esters of D-3-hydroxy-hexanoic acid (3-

OH-caproic acid, [redacted] monomer), DL-3-hydroxy-octanoic [redacted] (3-OH-caprylic acid, H8 monomer), DL-3-hydroxy-capric acid (H10 monomer), DL-3-hydroxy-lauric acid (H12 monomer) and DL-3-hydroxy-myristic acid (H14 monomer).

The transgenic plants expressing the PHA synthase C1 showed a significant increase in the size of the peaks corresponding to the H6-H14 monomers compared to the negative control plants. One novel peak was found only in PHAC1 plants and never in the negative controls. GC-MS was used to confirm that the peaks observed in both the PHAC1 plants and the negative controls were really identical to the standards and the novel peak was determined as being due to 3-hydroxy-octenoyl-methyl-ester containing a single unsaturated bond (H8:1 monomer). It is being speculated that the unsaturated bond is located at carbon 5 and has the cis conformation and that this monomer is due to the degradation of α-linolenic acid (18:3, all-cis, Δ9,12,15) and 16:3 (all-cis, Δ7, 10, 13) by β-oxidation. This reasoning is based on the prediction, that a D-3-hydroxy-octenoyl-CoA β-oxidation intermediate arises due to the cis-double bond at the even-numbered carbons (Gerhardt, B., *Lipid metabolism in plants* (Moore, T. S., Jr., ed.), CRC Press Inc., pp. 527-565 (1993)); see further discussions below under feeding studies). The same argument can be taken for the generation of the other monomers incorporated into the PHA, i.e. that they originated from fatty acids having a double bond at even-numbered carbons, which resulted in the formation of D-3-hydroxy-acyl-CoA β-oxidation intermediates. Thus the H8 monomer would originate from the degradation of linoleic acid (C18:2, all-cis, Δ9,12) or from C16:2, all-cis, Δ7, 10. This however does not satisfactorily explain the whole range of monomers observed, e.g. the H6 monomer would then have to originate from the fatty acids C18:1, Δ14-cis or C16:1, Δ12-cis, while the H14 monomer would have to originate from C18:1, Δ8-cis, or C16:1, Δ4-cis or C14:1, Δ2-cis, etcetera. As most of these would be rather uncommon fatty acids in *A. thaliana,* another argument for the origin of these PHA monomers can be proposed, which is based on the existence of an epimerase activity in plant β-oxidation (Preisig-Müller, R. et al., *J. Biol. Chem.* 269: 20475-20481 (1994)). In this case the D-3-OH-acyl-CoA β-oxidation intermediates are generated at a low rate by the "reverse" reaction catalyzed by the epimerase required for the conversion of D-3-hydroxy-acyl-CoA to the L-form, and sequestration of these D-intermediates into PHA actually

drives the reverse reaction. In this way the whole range of possible monomers can be explained, while the argument involving the unsaturated bond at even-numbered carbons in the acyl chains would still explain the relatively higher proportion of the H8-monomer and the existence of the H8:1 monomer.

Several negative control plants (both *A. thaliana* wild type and pART27 transgenic plants) were analyzed in various experiments without ever seeing more than only trace amounts of the various saturated monomers. The concentrations present in the negative controls were at least 1000 times smaller than in the positive plants, close to the detection limit of the methods at our availability. This was done by utilizing the GC-MS in the SIM mode (selected ion monitoring; ion 103 is characteristic for all of these 3-OH-fatty acid methyl esters) for which the detection limit was found to be approximately 4 pg/μL of the various standards. These compounds in the negative controls might also be intermediates of β-oxidation, i.e. mostly the L-3-hydroxy-acyl-CoAs and perhaps even very low amounts of the D-form, which are normally present at very low concentrations in the plant material in which β-oxidation is taking place. A rough calculation indicated a total PHA content of 0.03% (w/dry weight) in PHAC1#4.4 (multilocus plant), which related to approximately 5 μg of PHA in a large fresh leave weighing 155 mg. It was approximated that line PHAC1#3.3 produced 0.01% (weight/dry weight) in soil-grown plants.

EXAMPLE 5: Screening for PHA synthase C2 expressing plants

PHAC2 plants were screened directly for PHA production by analysis of dry leaves of T2 plants. Almost all of the T2 plants derived from 13 independently transformed plants were found to produce PHA in varying quantities, as judged by the presence of the novel peak due to the C8:1 monomer and also the peaks of the other PHA monomers. The highest producing plants were analyzed further and homozygous T3 plants were obtained. Two homozygous single-locus T3 lines were selected, PHAC2#19.5 and PHAC2#8.6. In comparison to PHAC1#3.3 plants, these PHAC2 plants produced slightly smaller quantities of PHA in seedlings grown on plates containing MS salts, kanamycin and sucrose. The

monomer composition of the respective transgenic plants was however identical. For that reason most of the further studies were only done with line PHAC1#3.3.

EXAMPLE 6: Immunolocalization and observation of PHA granules

For the immunolocalization of the peroxisomally-targeted PHA synthase C1, T3 seedlings of lines PHAC1#3.3 and pART27#21 (negative control) were grown on plates containing MS salts, kanamycin and sucrose. Seedlings were grown for 7 days under continuous light or in the dark after one day of illumination, the latter was done to obtain etiolated seedlings in which glyoxysomes are more abundant. The seedlings were fixed and sent together with some anti-PHA synthase C1 antiserum to Prof. Leech's laboratory at the University of York, where the immunolocalization was performed. It was found that the peroxisomes in PHAC1 seedlings were initially difficult to identify, since they did not look normal due to the presence of granules within them. These granules were very abundant in the etiolated seedlings, while in the light-grown seedlings most of the peroxisomes still looked normal or seemed to contain only tiny granules. The PHA synthase C1 was located in what seem to be two different types of organelles or peroxisomes, because the one contains a large quantity of PHA granules while the other contains apparently none. The darker peroxisomes without granules corresponded in appearance most closely to the normal peroxisomes in the negative controls. It is possible that this apparent heterogeneity is simply the results of non-homogenous distribution of granules within the peroxisomes. Glycolate oxidase was used as marker enzyme for peroxisomes of seedlings grown under light, while rubisco was used as chloroplastic marker. Antibodies against these two marker enzymes clearly identified the respective organelles in both PHAC1 seedlings and in the pART27 negative controls. Glycolate oxidase was found to be located in the organelles, i.e. the peroxisomes, containing PHA granules. Similarly the enzyme isocitrate lyase (ICL) was used as glyoxysomal marker in etiolated seedlings and it also confirmed that the granule-containing organelles were glyoxysomes. The antiserum against PHA synthase C1 unambiguously identified the peroxisomal localization of the PHA synthase in the PHAC1 seedlings, while it did not detect anything in the negative controls. Unusual accumulations of granules were also observed occasionally in the vacuoles of etiolated PHAC1 seedlings

and these globules were gold-labelled with anti-PHA synthase C1. This was in correspondence with the observation that the PHB synthase is found on the surface of PHB granules in bacteria (Gerngross, T. U. et al., *J. Bacteriol.* 175, 5289-5293 (1993)).

EXAMPLE 7: Changing PHA yield and monomer composition in feeding studies

Line PHAC1#3.3 was used to investigated if the total yield of PHA could be increased or if PHAs containing other monomers than the "native" PHA could be synthesized in PHAC1 transgenic plants. For that purpose seeds were sterilized and germinated in liquid medium containing mineral salts and 2% (w/v) sucrose supplemented with fatty acids or other compounds known to be degraded by β-oxidation. In experiment #1 the seedlings were grown for 3 days in the light before the substrates were added and the plant were moved into the dark. The material was harvested after 8 days and derivatized samples were analyzed by gas chromatography.

The results summarized in Table 1 point out several encouraging aspects. The yield of native PHA (obtained without feeding any substrate) was doubled when seedlings were germinated in the dark as opposed to continuous illumination. This could perhaps be ascribed to a more complete mobilization of the seed lipids in etiolated seedlings. In this respect the regulation of the glyoxylate cycle enzymes malate synthase and isocitrate lyase might play a role by affecting lipid-mobilization via β-oxidation. It has been shown that these glyoxylate cycle enzymes are regulated transcriptionally by three types of signal, namely light regulation, carbon catabolite repression by various sugars and developmental regulation during germination and senescence (Graham, I. A. et al., *Plant Mol. Biol.* 15: 539-549 (1990); Graham, I. A. et al., *Plant Cell* 4:349-357 (1992); Graham, I. A. et al., *Plant Cell* 6: 761-772 (1994)).

The large increase in the PHA yield obtained by the feeding of TWEEN-20 (Sigma; 50% palmitic acid (C16) esterified with polyoxyethylenesorbitol, the remainder is made up by lauric acid (C12) and myristic acid (C14) also esterified) (TWEEN is a registered trademark of ICI Americas, Inc., Wilmington, DE) indicated that the PHA synthase was

very active in these plants and thus not responsible for the relatively low yield of native PHA in seedlings grown without added fatty acids. The most pronounced effect of TWEEN-20 on the monomer composition was the decrease in the content of the H8:1 monomer from about 30% in native PHA to about 1%, which was most likely due to the lack of unsaturated fatty acid derivatives in the TWEEN-20. The relative distribution of the other monomers could be explained by the step-by-step β-oxidation of the C16, C14 and C12 components in TWEEN-20. A negative effect on seedling growth due to TWEEN-20 was observed, but it was small considering its high concentration (5% v/v) in the medium.

The accumulation of PHA granules in PHAC1 seedlings grown in liquid cultures supplemented with 5% TWEEN-20 under constant illumination for 12 days was very striking on electron microscope micrographs. These PHA granules were not observed in the negative controls, i.e. pART27 transgenic seedlings fed with TWEEN-20. The granules looked different from the starch granules observed in chloroplasts. These electron microscopic studies were done in our own institute by Mrs J. Petétot and the results confirmed similar results obtained with etiolated seedlings in Prof. Leech's laboratory.

TWEEN-60 (Sigma; 50% stearic acid (C18) and some palmitic and myristic acid; all esterified to polyoxyethylenesorbitol) and TWEEN-80 (Sigma; 50% oleic acid (C18:1), esterified to polyoxyethylenesorbitol) had less impact on the PHA yield, the monomer composition and the seedling growth than TWEEN-20. The relatively high level of the H8:1 monomer might be due to a higher contamination of TWEEN-60 and -80 with unsaturated fatty acids like α-linolenic acid, see above.

The free fatty acids hexanoate and octanoate were fed at very low concentrations due to their toxic effects on plant growth. For hexanoate a large increase of the H6 monomer was observed, while octanoate resulted in a very high increase of the H8 monomer together with a moderate increase in the H6 monomer. For both substrates the H8:1 monomer content remained relatively high, which was probably due to the normal accumulation of PHA from endogenous lipid β-oxidation ("native" PHA).

Table 1. Increasing total yield of PHA and changing its monomer composition in PHAC1 seedlings germinated in liquid media supplemented with fatty acids

Substrate	[sub] % (w/v)	light or dark (day 4 to 12)	fresh weight in mg[b]	mg PHA[a] per g fresh weight x 100^{-1}	% of total PHA (w/w)					
					H6	H8	H8:1	H10	H12	H14
None		light	232	1.9	1.3	43	29	10	9.7	6.8
None[c]		dark	186 ± 25	4.4	1.4	42	32	9.2	9.2	6.4
TWEEN-20	5	light	142	64	3.8	37	1.1	27	28	3.4
TWEEN-20[c]	5	dark	57 ± 24	70	4.0	41	1.5	25	25	2.9
TWEEN-60[c]	5	dark	125 ± 55	9.7	2.2	37	17	16	18	10
TWEEN-80[c]	5	dark	141 ± 34	6.5	3.2	44	20	15	14	4.0
Hexanoate (C6)[c]	0.05	dark	70 ± 3	11	30	32	21	7.0	7.4	3.1
Octanoate (C8)[c]	0.005	dark	125 ± 44	16	5.2	73	13	3.7	3.7	1.5

[a] The transesterified plant material (of specified weight) was in a volume of 1 mL chloroform, of which 1 µL was analyzed by GC.

[b] An average of 30 seedlings were grown per sample.

[c] Samples were done in duplicate and the results were averaged.

In experiment #2 (Tables 2 and 3) the seedlings were germinated for 8 days under continuous illumination, then the growth medium was replaced by the same medium containing 5% (v/v) TWEEN-80 together with various fatty acids, the purpose of the TWEEN-80 was to solubilize the water-insoluble fatty acids. The samples were placed back under constant illumination for another 6 days before being harvested and analysed. All samples were done in duplicate and each sample contained approximately 45 seeds which were germinated together in a large capped test-tube. Negative controls with pART27 plants were done for each substrate in the identical fashion. None of the novel PHA-monomer peaks were found in these negative controls.

Feeding of the saturated fatty acid tridecanoic acid (C13) and the branched fatty acid 8-methyl-nonanoic acid (8M-C9) resulted in the incorporation of a whole range of novel monomers. The identity of all these novel monomers was established by GC-MS. All of them had an uneven number of carbon atoms in their acyl chains and could be directly

traced to the original fatty acid supplied in the medium or intermediates of its degradation by β-oxidation. For tridecanoic acid, transgenic PHAC1 plants were found to contain a polymer having H13-, H11-, H9- and H7-3-hydroxy-alkanoic acid monomers. In the case of 8M-C9 the two novel monomers, 8-methyl-3-D-hydroxy-nonanoic acid (8M-H9) and 6-methyl-3-D-hydroxy-heptanoic acid (6M-H7), retained the branched structure of the original substrate. This shows that the PHA synthase C1 was able to incorporate a large variety of monomers into the polymer, provided that intermediates having the proper conformation were generated. The descending order in terms of quantities of the novel monomers (H13>H11>H9>H7; and 8M-H9>6M-H7) suggests that the β-oxidation of these unusual fatty acids proceeds slowly, thus permitting more time for intermediate-sequestration by the PHA synthase. It is possible that the 3-hydroxy-acyl-CoA dehydrogenase (MFP) and some other enzymes of the β-oxidation cycle have a low substrate specificity for these fatty acids and their derived intermediates.

Feeding of petroselenic acid (C18:1, 6-cis) resulted in a large increase in the content of the H14 monomer. This observation was in agreement with the proposed scheme of its degradation by β-oxidation (Gerhardt, B., *Lipid metabolism in plants* (Moore, T. S., Jr., ed.), CRC Press Inc., pp. 527-565 (1993)). All unsaturated bonds in the cis-conformation starting at an even-numbered carbon in the acyl chain were proposed to present obstacles to the normal cycle of the β-oxidation and had to be circumvented by modifications of the pathway. This is because the D-3-hydroxy-acyl-CoA can be formed by the action of the enoyl-CoA hydratase (MFP) from 2-cis-enoyl-CoA (cis-unsaturated bond in even-numbered position), but the D-3-hydroxy-acyl-CoA cannot be utilized by the 3-hydroxy-acyl-CoA dehydrogenase (MFP), which can only act on the L-3-hydroxy-acyl-CoA. Three possible modifications were put forward: 1) An epimerase converts the D-3-hydroxy-acyl-CoA to the L-form. 2) A dehydratase (also called D-3-hydroxyacyl-CoA hydrolyase or D-specific 2-trans-enoyl-CoA hydratase II, see Engeland, K. and Kindl, H., *Eur. J. Biochem.* 200: 171-178 (1991)) converts the D-3-hydroxy-acyl-CoA to 2-trans-enoyl-CoA, which can then be reconverted to L-3-hydroxy-acyl-CoA by the enoyl-CoA hydratase I. 3) A 2,4-dienoyl-CoA reductase reduces the 2-trans-4-cis-acyl-CoA β-oxidation intermediate to the 3-cis-enoyl-CoA, which in turn will require the activity of an isomerase to form the 2-trans-enoyl-

CoA β-oxidation intermediate. The first two options would result in the generation of D-3-hydroxy-acyl-CoA intermediates which would be directly available to the PHA synthase. Thus the observation of the specific increase in the H14 monomer upon feeding with petroselenic acid fits well with the predicted modifications of the β-oxidation to bypass the cis-unsaturated bond at carbon 6 of petroselenic acid. The same modifications have also been used above to explain the presence of the 3-hydroxy-octenoyl monomer (H8:1) in the native PHA. It was speculated that this monomer was due to the degradation of 18:3, all-cis-Δ9, 12, 15 and 16:3, all-cis-Δ7, 10, 13 by β-oxidation. The high proportion of H8 monomer could similarly be due to the degradation of linoleic acid (18:2, all-cis-Δ9,12) which is an abundant fatty acid in plant material.

The degradation of fatty acids containing hydroxy groups on even-numbered carbon atoms in either the D- or the L-conformation also poses obstacles to the normal β-oxidation pathway and modifications are required to bypass these (Gerhardt, B., *Lipid metabolism in plants* (Moore, T. S., Jr., ed.), CRC Press Inc., pp. 527-565 (1993)). The D-4-hydroxy-decanoate-CoA and D-2-hydroxy-octanoate-CoA intermediates were predicted to arise in the degradation of ricinoleic acid (D-12-hydroxy-oleic acid (9-cis)). To investigate whether these intermediates might be incorporated into the PHA polymer by the PHA synthase, ricinoleic acid was used to supplement the medium in which PHAC1 plants were germinating. No major peaks due to the incorporation of novel monomers into the PHA polymer were detected, but GC-MS analysis was utilized to search for specific predicted novel monomers by looking for characteristic fragmentation products, namely ions 117 and 89. A small peak was found with ion 117, this peak showed the fragmentation fingerprint of the D-4-hydroxy-decanoate-methyl ester and was absent in the corresponding negative control. No novel peak was found with ion 89, thus excluding the possibility that the D-2-hydroxy-octanoate was incorporated into the polymer. It is known that the PHA synthase can incorporate D-4-hydroxy- and D5-hydroxy monomers into PHA in bacterial cultures, therefore the incorporation of the D-4-hydroxy-decanoate in the germinating seeds fed with ricinoleic acid was plausible. The very low abundance of the monomer could perhaps be explained by an alternative and more efficient pathway for the degradation of ricinolate

(Gerhardt, B., *Lipid metabolism in plants* (Moore, T. S., Jr., ed.), CRC Press Inc., pp. 527-565 (1993)).

Table 2. Quantity of PHA production in PHAC1 seedlings germinated in liquid medium supplemented with fatty acids

Substrate	[Substrate] % (w/v)	fresh weight (mg)	mg PHA[a] per gram fresh weight x 0.01
None		458 ± 8	4.6
5% TWEEN-80 (T)		549 ± 43	5.0
Tridecanoic acid (C13) + T	0.1	276 ± 9	28
8-methyl-nonanoic acid (8M-C9) + T	0.1	48 ± 14	46
Petroselenic acid (C18:1, 6-cis) + T	1	287	9.4
Ricinoleic acid (D12-OH-C18:1, 9-cis) + T	0.1	215 ± 21	6.0

[a] The plant material (of specified weight) was transesterified in different volumes, but the integrated peak-areas were calculated to homologate the sample-volumes (1 mL chloroform, of which 1 µL was analyzed by GC).

Table 3. Monomer composition of PHA produced in PHAC1 seedlings germinated in liquid medium supplemented with fatty acids

Substrate	% of total PHA (w/w)												
	H6	H7	6M-H7[a]	H8	H8:1	H9	8M-H9[a]	H10	4OH-H10[b]	H11	H12	H13	H14
None	1.0			36	28			10			14		10
5% TWEEN 80 (T)	2.1			44	14			19			15		6.3
Tridecanoic acid (C13) + T	0.46	9.6		9.1	2.4	18		3.4		20	3.2	32	1.5
8-methyl-nonanoic aci (8M-C9) + T	0.20		24	9.2	3.0		55	3.0			2.8		2.2
Petroselenic acid (C18:1 6-cis) + T	1.6			32	7.4			17			17		26
Ricinoleic acid (D12 OH-C18:1, 9 cis) + T	1.8			46	8.8			30	1.2[c]		7.4		5.0

[a] 8M-H9 and 6M-H7 refer to 8-methyl-3-D-hydroxy-nonanoic acid and 6-methyl-3-D-hydroxy-heptanoic acid, respectively.

[b] 4-OH-H10 refers to D-4-hydroxy-decanoate.

[c] The quantity of 4-OH-H10 was estimated by comparing peak sizes with H6 on a GC-MS chromatogram.

EXAMPLE 8: Extraction of high molecular weight PHA

The presence of derivatized monomers of PHA in PHAC1 plants had been established by the GC-analysis of trans-esterified intact plant material. To prove that the PHA was synthesized as high-molecular weight polymer and for its physico-chemical characterization, the purification of large quantities (i.e. in the mg range) was undertaken. Seeds of PHAC1#3.3 were germinated in liquid medium with and without addition of TWEEN-20 in order to obtain TWEEN-20-derived PHA or unmodified PHA, respectively.

For the TWEEN-20-derived PHA, approximately 16000 seeds (313 mg dry seeds) were germinated in 900 mL 1/2xMS + 1% sucrose medium for 7 days under continuous illumination on a shaker, the medium was replaced with 1/2xMS + 2% sucrose containing 5% TWEEN-20 and the seedlings were grown for another 9 days in the light. The plant material was harvested, washed extensively with water to remove residual TWEEN-20, frozen and lyophilized. The dry material was ground with a mortar and pestle, weighed, and lipids were extracted by a six-hour Soxhlet-extraction with methanol. The methanol-insoluble PHA was extracted for 24 hours in the same manner with chloroform. The chloroform extract was concentrated under reduced pressure and the PHA was precipitated by the addition of 10 volumes of cold methanol. This methanol precipitation was performed twice to ensure a high purity of the PHA. 27 mg of PHA was thus obtained from 5.35 g lyophilized and powdered seedling material, which related to 0.50% weight/dry weight. The PHA was trans-esterified and analyzed by GC. It was found that 58% of the PHA present in the methanol-extracted plant powder was extracted by the chloroform. It has been established in previous experiments that this remaining PHA was recalcitrant to extraction. The chromatogram showed that the extracted PHA was adequately pure with the peaks of the six identified monomers constituting 93% of the total integrated area. The ratio of the integrated areas between the different monomers was very similar to the result shown in Table 1 for the sample containing TWEEN-20 and grown under light, see Table 4.

For the extraction of high-M_r PHA produced by PHAC1 plants without additional fatty acid supplements (native PHA), 1076 mg seeds (approx. 54000 seeds) were germinated in 3.3 L liquid medium (1/2xMS, 2% sucrose). The seeds were germinated under continuous illumination for 6 days, thereafter the medium was replaced and the seedlings put into the dark for another 7 days in order to induce plant senescence. The PHA was extracted from the plant material as above and one methanol precipitation was performed to purify the PHA. 23 mg of PHA was obtained from 14.3 g dry plant material, which related to 0.16 % weight/dry weight. It was determined that ≥69 % of the PHA had remained in the plant material after the chloroform extraction, which could be due to either the high content of C8:1 monomer (see Table 5) causing the polymer to "stick", or due to moisture in the ground material which had not been lyophilized completely, or due to the

large sample size for which a longer and more efficient chloroform extraction might have been required. The purification of native PHA and analysis by GC-MS allowed the detection of several more peaks that could not be initially resolved in crude extracts because of the high level of noise in the chromatogram. A total of eighteen 3-hydroxyacid monomers could be detected in the polymer (Table 1). In addtion to 3-hydroxyhexanoic acid (H:6), 3-hydroxyoctanoic acid (H:8), 3-hydroxydecanoic acid (H:10), 3-hydroxydodecanoic acid (H:12), 3-hydroxytetradecanoic acid (H:14) and 3-hydroxyoctenoic acid (H8:1) monomers previously detected in the transesterification of intact plant material (crude extract) (Table 1), novel saturated and unsaturated monomers were detected which include 3-hydroxyhexadecanoic acid (H:16), 3-hydroxynonanoic acid (H9), 3-hydroxyundecanoic acid (H:11), 3-hydroxytridecanoic acid (H:13), 3-hydroxyhexadecatrienoic acid (H16:3), 3-hydroxyhexadecadienoic acid (H16:2), 3-hydroxyhexadecenoic acid (H16:1), 3-hydroxytetradecatrienoic acid (H14:3), 3-hydroxytetradecadienoic acid (H14:2), 3-hydroxytetradecenoic acid (H14:1), 3-hydroxydodecadienoic acid (H12:2) and 3-hydroxydodecenoic acid (H12:1). All even-chained monomers could be quantified and results are shown in Table 5.

It is expected that many of the unidentified minor peaks detected in the PHA purified from the TWEEN-20-fed seedlings would correspond to some of the minor saturated and unsaturated monomer detected in the "native" PHA.

Table 4. Comparison of the monomer composition of purified high-molecular weight PHA from Tween-20 feed plants with results obtained for transesterified intact seedlings during the preliminary feeding studies

Sample		% of total PHA area[a]					
		H6	H8	H8:1	H10	H12	H14
Purified high Mr PHA	TWEEN-20 derived	1.9	33.5	0.50	29	32	2.7
TWEEN-20 + light	see Table 1, line 3	3.8	37	1.1	27	28	3.

[a] Integrated area on the chromatogram.

Table 5. Monomer composition of "native" PHA isolated from *phaC1*-transformed plant line 3.3 grown in liquid media[a]

Monomer	H6	H8	H8:1	H10	H12	H12:1	H12:2
% (w/w)	1.1	23	18	4.7	5.8	4.3	5.0
std. dev.	0.16	4.4	4.6	0.51	0.46	0.60	1.3
Monomer	H14	H14:1	H14:2	H14:3	H16	H16:2	H16:3
% (w/w)	4.2	6.7	7.5	11	2.0	2.0	5.6
std. dev.	1.1	2.3	1.4	3.2	0.26	0.41	1.3

[a] Quantification of methyl esters was performed with a GC with a FID detector. Values were obtained from four separate PHA preparations. Monomers present in trace amounts (H9, H:11, H:13, H16:1) were not quantified.

EXAMPLE 9: Chemical characterization of high-molecular weight plant PHA

Purified TWEEN-20-derived PHA (13 mg) and unmodified PHA (5 mg) were given to Géraldine Coullerez at the EPFL (collaboration IBPV-EPFL) for the physico-chemical characterization of the polymer. Two different samples of bacterial PHA, PHA1 and PHOE, were obtained from Witholt and Kellerhals (ETH Zürich) to be used as controls. PHA1 contained predominantly H6 and H8 monomers (10% and 90%, respectively), while PHOE contained 4-10% H8:1, the balance being H6 and H8. The molecular weights and the respective dispersion coefficients of the polymers were determined by gel permeation chromatography (see Table 6). Polystyrene polymers were used as molecular weight standards. The results clearly show that the TWEEN-20 derived PHA produced by the transgenic plants is in the form of a high-M_r polymer (about 200-250 monomers), although the molecular weight is only 20-25% of the bacterial polymers (about 1000 monomers). This shorter polymer length can be explained by an overabundance of PHA synthase relative to its substrate concentration and similar results have also been obtained in *in vitro* polymerization assays with purified PHB synthase (Jun Sim, S. et al., *Nature Biotechnology* 15: 63-67 (1997)). It is also possible that PHA polymers with longer chain lengths are trapped in the plant material, since a significant proportion of the PHA seems to be

recalcitrant to chloroform extraction (≥ 50%, difficult to determine exact amounts in the trans-esterification of intact or powderized plant material, see above).

NMR analysis of the plant and bacterial PHAs revealed, that the TWEEN-20 derived plant PHA had the same structure as the bacterial PHA. The NMR spectrum of the unmodified plant PHA showed the peaks characteristic for the PHA polymer backbone, as well as several other peaks which have not been properly assigned or identified at this stage, but which could be due to various unsaturated bonds in the side chains of the polymer.

Table 6. Comparison of molecular weights of high-Mr PHA_{mcl} purified from plants and bacteria

Origin of PHA	Mw	Mn	Dispersion Index
TWEEN-20 derived PHA - *Arabidopsis* PHAC1#3.3	4.01×10^4	4910	8.17
PHA1 from *Pseudomonas oleovorans*	1.46×10^5	58850	2.48
PHOE from *P. oleovorans*	2.0×10^5	81590	2.44

EXAMPLE 10: The multifunctional protein (MFP) from the yeast *Candida tropicalis*

In animals, plants and bacteria, β-oxidation has been shown to proceed via the L-isomer of the 3-hydroxy-acyl-CoA intermediates and any D-isomers (which are predicted to arise in the degradation of fatty acids containing cis-unsaturated bonds at even-numbered carbons) have to be converted to the L-form in order to be oxidized further by the dehydrogenase activity of the multifunctional protein (MFP). In yeast the β-oxidation was reported to proceed via the D-isomer (Nuttley, W. M. et al., Gene 69: 171-180 (1988); Hiltunen, J. K. et al., *J. Biol. Chem.* 267: 6646-6653 (1992); Fosså, A. et al., *Mol. Gen. Genet.* 247: 95-104 (1995)). The yeast multifunctional protein (MFP) was shown to contain enoyl-CoA hydratase II and D-3-hydroxyacyl-CoA dehydrogenase activities, which together converted trans-2-enoyl-CoA via D-3-hydroxyacyl-CoA to 3-ketoacyl-CoA, i.e. the D-isomer was directly utilized by the dehydrogenase without prior conversion to the L-form.

It is anticipated that expression of this hydratase II activity together with the PHA synthase in the peroxisomes of double-transgenic plants will generate more of the D-3-hydroxy-acyl-CoA intermediates for their incorporation by the PHA synthase into the PHA polymer, thus increasing the final yield of PHA. Four separate approaches are envisioned.

A. Expression of the unchanged MFP from *C. tropicalis* in *A. thaliana.*

Since the hydratase II activity forms part of the MFP it was decided to perform investigatory experiments with the complete MFP prior to attempting to abolish the D-3-hydroxyacyl-CoA dehydrogenase activity. As the fungal MFP already had a peroxisomal targeting signal, this protein was expected also to be targeted to the plant peroxisomes.

The *C. tropicalis* MFP cDNA (Nuttley, W. M. et al., *Gene* 69: 171-180 (1988), GenBank Accession Number M22765) was cloned via PCR amplification (SEQ ID NO:21, encoding SEQ ID NO:22) into pART7 to obtain pART7_MFP. The NotI-cassette, containing the CAMV35S-promoter in front of the MFP gene and the ocs3'- terminator, was inserted into the plant binary vector pART27 to obtain pART27_MFP, which was transformed into *Arabidopsis*. Transgenic plant were selected on kanamycin and screened for the expression of the MFP protein with an anti-MFP antiserum. Homozygous T2 plants were cross-fertilized with PHAC1#3, PHAC1#4 and PHAC1#9 plants. Offspring from these crosses will be analyzed for their ability to biosynthesize PHA.

B. Changing the peroxisomal targeting signal of the yeast multifunctional protein (MFP) from -AKI to -SKL.

The COOH-terminal tripeptide -AKI was shown to be responsible for peroxisomal targeting of the MFP in yeast, but it has not yet been demonstrated to function in plant peroxisomal targeting. The MFP.SKL gene, in which the 3'-terminal nucleotide sequence of the MFP gene encoding the -AKI tripeptide had been changed to -SKL by PCR site-directed mutagenesis (SEQ ID NO:23, encoding SEQ ID NO:24), was obtained from the laboratory of K. Hiltunen to ascertain that the MFP was properly targeted to the plant peroxisomes and

to serve as a positive control in targeting studies with the yeast multifunctional protein (MFP) in plant cells. The MFP.SKL gene was used to construct pART7_MFP.SKL. The NotI-cassette of pART7_MFP.SKL, containing the MFP-SKL gene flanked by the CaMV35S promoter and the ocs3'-terminator, was cloned into pART27 to obtain pART27_MFP.SKL, which was transformed into *A. thaliana* ecotype Columbia. Kanamycin resistant T1 plants were obtained. The high-MFP.SKL-expressing lines will be selected by Western analysis of T2 plants, and the selected lines will be crossed with PHAC1#3.3 plants.

C. Deleting the peroxisomal targeting signal of the yeast multifunctional protein (MFP).

The construct pART7_MFPΔAKI was obtained by PCR amplification of the MFP gene such that the 3'-terminal nucleotide sequence of the MFP gene encoding the -AKI tripeptide was deleted by the introduction of a stop codon (SEQ ID NO:25, encoding SEQ ID NO:26). The "detargeted" MFPΔAKI is expected to be localized in the cytoplasm and will be utilized as negative control in experiments to study the localization of MFP and MFP.SKL in plant cells. pART27_MFPΔAKI was transformed into *A. thaliana* ecotype Columbia and Kanamycin resistant T1 plants were obtained. The high-MFPΔAKI-expressing lines will be selected by Western analysis of T2 plants and these lines will be crossed with PHAC1#3.3 plants.

D. Deleting the dehydrogenase activity of the yeast multifunctional protein (MFP).

As only the hydratase II activity of the yeast multifunctional protein (MFP) is of interest, plants will be transformed with the MFPΔDH gene, in which the dehydrogenase activity was deleted by site-directed mutagenesis of specific amino acid residues identified as being essential for this activity.

EXAMPLE 11: Verification of enzyme activity of modified MFP constructs in *Pichia*

The modified MFP.SKL and MFPΔAKI genes were subcloned from pART7_MFP.SKL and pART7_MFPΔAKI into the yeast expression vector pHILD2. The resulting plasmids pHILD2_MFP.SKL and pHILD2_MFPΔAKI were transformed into *Pichia* and enzyme assays were performed in Hiltunen's laboratory. Results indicated that the modifications to the genes did not have an effect on the dehydrogenase and the hydratase enzymatic activities.

EXAMPLE 12: Expression of the FatB3 acyl-ACP thioesterase in double transgenics to increase PHA yield

Expresion of the California bay acyl-ACP thioesterase was shown to cause premature termination of fatty acid elongation during fatty acid biosynthesis in transgenic oilseed plants (Voelker, T. A. et al., *Science* 257: 72-74 (1992)). The resulting medium-chain-length fatty acids were found to accumulate in the triglycerides of seed lipids, but could not be detected in leaves. It is thought that medium chain fatty acids do not accumulate in the leaves of transgenic plants because they get degraded immediately by β-oxidation (Eccleston, V. S. et al., *Planta* 198: 46-53 (1996)). This increased flux of medium-chain fatty acids through β-oxidation may be exploited to improve the yield of PHA, as well as to modify the composition of the polymer towards saturated H6-H14 monomers in double transgenic plants expressing both acyl-ACP thioesterase and the PHAC1 synthase.

The plasmid pBJ49_FatB3 containing the *Cuphea lancolata* thioesterase FatB3 gene under control of a 200 bp minimal promoter derived from the 35S promoter was infiltrated into the *A. thaliana* PHAC1#3.3 transgenic line which is homozygous for the PHAC1 gene. Hygromycin resistant lines where obtained and the seed lipid content of T1 seeds was analysed for increased levels of medium chain length fatty acids and 11 separate lines expressing high levels of the acyl-ACP thioesterase were identified in this manner. Subsequently the polyhydroxyalkanoate content of leaves from soil grown T2 double transgenic offspring was determined by GC and GC-MS analysis of the 3-hydroxy-fatty

acid methyl esters obtained by transesterification of whole leaves. The results (Table 7) indicated an approximate tenfold increase in the polyhydroxyalkanoate content of leaves from double transgenic plants when compared to plants expressing only the PHAC1 synthase. The increased polyhydroxyalkanoate yield was mainly due to a large increase in the content of the saturated polyhydroxyalkanoate monomers with an even number of carbons, namely 3-OH-octanoate (H8), 3-OH-decanoate (H10), 3-OH-dodecanoate (H12) and 3-OH-tetradecanoate (H14) (Table 8).

The recombinant FatB3 acyl-ACP thioesterase is naturally targeted to the chloroplast, where it removes medium chain-length acyl-ACP intermediates from the fatty acid biosynthesis. These short chain fatty acids accumulate in the seed lipids, but not in the leaves of transgenic plants and it has been speculated, that they are immediately degraded by β-oxidation. Results with these double transgenic plants indicate that there is indeed an increase in the β-oxidation of medium chain length fatty acids in the leaves, which results in a higher yield of polyhydroxyalkanoate due to the incorporation of the β-oxidation intermediates into the PHA by the polyhydroxyalkanoate synthase.

Table 7. PHA content of leaves from single and double transgenic plants expressing the PHAC1 synthase alone or together with the FatB3 acyl-ACP thioesterase

Plants	PHA content (mg/g fresh weight)		
		average	std deviation
PHAC1#3.3 plant 1	0.0040		
PHAC1#3.3 plant 2	0.0253	0.0147	0.015
PHAC1#3.3 + FatB3 line 2.4a plant 2	0.1281		
PHAC1#3.3 + FatB3 line 2.4b plant 1	0.0749	0.1175	0.038
PHAC1#3.3 + FatB3 line 2.4b plant 5	0.1495		

Table 8. PHA content of leaves from single and double transgenic plants expressing the PHAC1 synthase alone or together with the FatB3 acyl-ACP thioesterase

Monomer	PHAC1#3.3			PHAC1#3.3 + FatB3		
	mg/g	std. dev.	% (w/w)	mg/g	std. dev.	% (w/w)
H6	0.00035	0.00036	2.39	0.00455	0.00131	3.87
H8:1	0.00451	0.00525	30.76	0.00790	0.00913	6.73
H8	0.00205	0.00201	13.94	0.03765	0.01120	32.05
H9	0.00014	0.00001	0.95	0.00029	0.00010	0.25
H10	0.00087	0.00080	5.91	0.04694	0.01816	39.96
H11	0.00017	0.00002	1.17	0.00034	0.00015	0.29
H12	0.00145	0.00145	9.87	0.00642	0.00247	5.47
H13	0.00016	0.00010	1.09	0.00023	0.00013	0.20
H14:1	0.00072	0.00059	4.92	0.00141	0.00114	1.20
H14:2	0.00121	0.00142	8.21	0.00179	0.00209	1.53
H14:3	0.00086	0.00106	5.86	0.00142	0.00178	1.20
H14	0.00219	0.00222	14.93	0.00853	0.00459	7.26

EXAMPLE 13: Crossing PHAC1#3.3 transgenic plants with fatty acyl hydroxylase LFah12 transgenic plants

Three lines of transgenic *A. thaliana* expressing the LFah12 fatty acyl hydroxylase gene from *Lesquerella* were obtained from Pierre Broun (Chris Somerville's laboratory, Carnegie Institution, Stanford, CA). This fatty acyl hydroxylase is responsible for the production of ricinoleic acid (C18:1; 9-cis, D-12-hydroxy) in *Lesquerella*. It was found that hydroxylated fatty acids accumulated in the seed triglycerides of *Arabidopsis*, but not in the leaves, again indicating that hydroxylated fatty acids synthesized in leaves are most likely degraded by β-oxidation (Broun, P. and Somerville, C., *Plant Physiol.* 113: 933-942 (1997); van de Loo, F.N. et al., *Proc. Natl. Acad. Sci. U.S.A.* 92: 6743-6747 (1995)). Crosses were made with the three fatty acyl hydroxylase transgenic lines and the PHAC1#3.3 line and the seeds of these crosses were harvested. Seeds and their progeny plants will be examined for their levels of PHA biosynthesis. The aim of this experiment is to investigate if the increased flux of hydroxylated fatty acids to the β-oxidation cycle in transgenic plants expressing the Fah 12 and PHA synthase genes can lead to an increase in the yield of PHA and if novel hydroxylated monomers can be incorporated in the PHA.

EXAMPLE [redacted] Influence of carbon source and light co[redacted]itions on PHA synthesis

The amount of PHA present in plant tissues was influenced by the growth conditions . For plants grown for three weeks under constant illumination in MS liquid media with 2% sucrose, the yield of PHA was approximately 0.6 mg/g dry weight (dwt). Removal of sucrose for the last week of growth in the light resulted in a 100% increase in PHA, while plants growing in 2% sucrose but shifted in the dark for the last week accumulated 22% more PHA (Table 9).

Table 9. Influence of sucrose and light on PHA accumulation in phaC1-transformed line 3.3

PHA yield	Growth conditions[a]					
	0% sucrose	0.2% sucrose	2% sucrose	0% sucrose	0.2% sucrose	2% sucrose
	dark	dark	dark	light	light	light
mg PHA/g dwt	1.42	1.31	0.73	1.23	1.08	0.60
Relative %[b]	100	92	52	87	76	42

[a] Seedlings were grown under constant illumination in a liquid medium containing MS salts and 2% (w/v) sucrose for 2 weeks, and then grown for another week, either in the dark or in the light, in media containing different concentrations of sucrose.
[b] The yield of 1.42 mg/g dry weight was arbitrarily defined as 100%.

EXAMPLE 15: Peroxisome targeting

It has been shown in multiple sytems (e.g., yeast, animal, and plants) that targeting of proteins to the peroxisome can be acheived by the addition of as little as three amino acids at the carboxy end of a foreign protein (see Gietl, C., *Physiol. Plant.* 97: 599-608 (1996); Purdue, P.E. and Lazarow, P. B., *J. Biol. Chem.* 269: 30065-30068 (1994); Subramani, *Ann. Rev. Cell Biol.*, 9: 445-478 (1993)). The minimal consensus sequence for peroxisome targeting of protein via the carboxy end, named PTS1 for peroxisomal targeting sequence 1, is a small uncharged amino acid at position 1 (S, A, or P), a positively-charged

amino acids at positi (K, R, S, or H), and a hydrophobic a acid at position 3 (L, M, I or F).

Thus, although the initial minimal PTS1 sequence was defined as SKL, a range of substition have been found to be effective PTS1 signal, including ARM, SRM, SKL, ARL, SRL, PSI, or PRM. Specific examples of targeting of foreign proteins in plants include: 6 amino acid PTS1 (RAVARL, Volokita, M., *Plant J.*1: 361-366 (1991)); 5 amino acids PTS1 (AKSRM, Olsen, L. J. et al, *Plant Cell* 5: 941-952 (1993)); 4 amino acids PTS1 (KSRM, Trelease, R. N. et al., *Protoplasma* 195: 156-167 (1996)); 5 amino acid PTS1 (ELSRL, Hayashi, M et al, *Plant J.* 10: 225-234 (1996)); 4 amino acid PST1 (RPSI, Mullen R. T. et al, *Plant J.* 12: 313-322 (1997)); 3 amino acid PTS1 (SKL, Banjoko, A. et al., *Plant Physiol* 107: 1201-1208 (1995)); 3 amino acid PTS1 (ARM, Lee, M.S. et al., *Plant Cell* 8: 185-197 (1997)).

A comparison of the peroxisomal targeting sequence 1 (PTS1) found in mammals, fungi and trypanosomes was performed by Purdue, P.E. and Lazarow, P.B. (*J. Biol. Chem.* 269: 30065-30068 (1994). All sequences shown in Table 10 are functional in at least one species. Other sequences may or may not have been tested. For trypanosomes, all sequences with a single amino acid change from SKL that are not shown are nonfunctional. The asterisks refer to the fact that -NKL and -SQL (outside the mammalian consensus, but not directly tested) have been found at the C termini of mammalian peroxisomal proteins. Uppercase, functional; lowercase, nonfunctional; underlined, not yet found on a peroxisomal protein in that species.

Table 10. C-termi[redacted]eroxysomal targeting sequences.

Mammals	*S. cerevisiae*	*H. polymorpha*	*C. albicans*	Trypanosomes
SKL	SKL	SKL		SKL
SRL				SRL
SHL				SHL
AKL				AKL
CKL				CKL
skf	SKF			
ski		SKI		SKI
*		NKL		NKL
		ARF		
	AKI		AKI	
	aqi		AQI	
	gki		GKI	
ssl				SSL
				SKM
tkl				G/H/P/T-KL
*				S-M/N/Q-L
				SKY

The minimal peroxisomal targeting sequence 1 (PTS1) in plants has been found to be ARM, SRM, SKL, ARL, SRL, PSI, and PRM (Compilation from Volokita, M., *Plant J.*, 1: 361-366 (1991); Olsen, L.J. et al., *Plant Cell*, 5: 941-952 (1993); Trelease, R.N. et al., *Protoplasma*, 195: 156-167 (1996); Gietl, C., *Physiol. Plant.*, 97: 599-608 (1996); Purdue, P.E. and Lazarow, P.B., *J. Biol. Chem.*, 269: 30065-30068 (1994); Subramani, *Ann. Rev. Cell Biol.*, 9:445-478 (1993); Mullen, R.T., et al., *Plant J.*, 12: 313-322 (1997); Lee, M.S., et al., *Plant Cell*, 9: 185-197 (1997)).

Some proteins are targeted to the peroxisome via an N-termianl extension called PTS2 for peroxisome targeting sequence 2. In this case, a consensus sequnce of nine amino

acids has been de[redacted]d, being (R/K)(L/Q/I)XXXXX(H/Q)[redacted] Foreign protein (eg β-glucuronidase) can also be targeted in plants to the peroxisome by adding a PTS2 sequence at the N-terminal end of the protein (Kato et al, *Plant Cell* 8: 1601-1611 (1996)).

EXAMPLE 16: Co-expression of PHA with other sequences resulting in increased or novel PHA biosynthesis

PHA_{mcl} synthesized in transgenic plants can include a large variety of monomers, with functional groups that can be used to modify and improve the characteristics of the polymer before or after extraction form the plant. For example, the presence of double bonds, epoxy groups, or acetylated groups within the PHA may be used to cross-link the polymer. The examples herein have demonstrated the incorporation of the following range of monomers into plant PHAmcl: even-chain saturated 3-OH-acyl monomers with six to sixteen carbons; odd-chain saturated 3-OH-acyl monomers with seven to thirteen carbons; unsaturated 3-OH-acyl monomer with 8, 12, 14, and 16 carbons and with 1, 2, or 3 double bonds; branched-chain 3-OH-acyl monomers (8-methyl-3-D-hydroxy-nonanoic acid and 6-methyl-3-D-hydroxy-heptanoic acid) and 4-OH-acyl monomers (D-4-hydroxy-decanoate). Although in these experiments some monomers, such as branched-chain, odd-chain or hydroxylated 3-hydroxyacids, were found included in PHAs after exogenous fatty acids were supplied to the transgenic plants, the same range of monomers would also be included in plant PHA from fatty acids supplied from endogenous fatty acid synthesis. Thus, one can predict being able to synthesize PHA polymers in plants that have a wide range of monomers, for example, higher proportion of short-chain monomers, unsaturated bonds at novel positions, monomers with hydroxylated groups, epoxy groups, acetylated groups, keto groups, cyclopentenyl groups, cyclopropanoid groups, furanoid groups or halogenated groups, branched chain, cyclic groups or any other novel monomers for which the equivalent functional groups exist in fatty acids in plants. The incorporation of these novel monomers derived from fatty acids into plant PHAs could be accomplished by expressing a PHA synthase in a plant which synthesizes these unusual fatty acids either naturally or after expression of a transgene such as fatty-acyl-thioesterases, -hydroxylases, -desaturases, -epoxidases, or -acetylases.

It is also conceivable that the substrate specificity of the PHA synthase could be modified to allow the incorporation of a wider range of monomers into PHA. One can predict that the range of monomers which could be included into plant PHAs from such a modified PHA synthase will include monomers that can be derived from plant fatty acid metabolism found in wild type plants or plants expressing transgenes (such as desaturases, hydroxylases, thioesterases, epoxydases, acetylases) which results in the modification of fatty acids synthesized in plants. It is also conceivable that suitable hydroxy acid substrates for the PHA synthase can be obtained from the amino acid metabolism or the plant secondary metabolism.

It has been demonstrated before that plants can synthesize PHB from acetyl-CoA through the expression of the 3-ketothiolase, acetoacetyl-CoA reductase and PHB synthase from *A. eutrophus* (Poirier, Y. et al., *Science* 256: 520-523 (1992); Nawrath, C. et al., *Proc. Natl. Acad. Sci. U.S.A.* 91: 12760-12764 (1994)). The examples herein demonstrate that PHA_{mcl} can be synthesized in plants expressing a PHA synthase which can accept monomers from H6-H16. Since acetyl-CoA is also found in the peroxisome, one can predict that co-expression of a PHA synthase with a substrate specificity for 3-hydroxyacids ranging from H4 to H8 or higher in the peroxisome, and of the *A. eutrophus* acetoacetyl-CoA reductase, would lead to the biosynthesis of a copolymer containing hydroxybutyrate and hydroxyacids of H6 and higher. In this pathway, the expression of the 3-ketothiolase from *A. eutrophus* may not be required since the peroxisome already contains a 3-ketothiolase.

The examples herein clearly show that synthesis of PHA in plants can be significantly enhanced by increasing the pool of fatty acids which is channeled through β-oxidation. Thus, when short-chain fatty acids were added externally in the form of TWEEN-20 to PHAC1-transgenic plants, there was a 30- fold increase in the amount of PHA synthesized in plants. Similar large increases in PHA synthesis were found when tridecanoic acid and 8-methyl-nonanoic acid were added to the growth media. It is hypothesized that because these fatty acids could not be incorporated into membranes without disrupting them, the fatty acids are detoxified by channeling them to the peroxisome

for degradation by the β-oxidation cycle. Thus, increased channeling of fatty acids to the β-oxidation cycle results in an increase in PHA synthesized using intermediates of fatty acid oxidation. One can predict from this work that any changes in plants which results in an increased flux of fatty acids to the β-oxidation cycle will results in an increase in PHA synthesis in plants expressing a PHA synthase targeted to the peroxisome. Increasing the flux of fatty acids to the β-oxidation cycle could be accomplished by overexpressing enzymes which lead to the biosynthesis of modified fatty acids. This has been demonstrated in plants expressing thioesterase (Eccleston, V.S. et al., *Planta* 198: 46-53 (1996)) and implied in plants expressing hydroxylase (van de Loo, F.N. et al., *Proc. Natl. Acad. Sci. U.S.A.* 92: 6743-6747 (1995)). Increase of flux of lipids to the β-oxidation cycle and to PHA synthesis could also be accomplished by expressing other fatty acid modifying enzymes, such as desaturases, epoxydases, acetylases, enzymes involved in synthesis of branched-chain fatty acids, etcetera. This concept has been directly demonstrated in this present work with a fatty acyl-ACP thioesterase. It was shown that co-expression of a fatty acyl-ACP thioesterase in a plant expressing a peroxisomal PHA synthase leads to a 10 fold increase in PHA (Table 7). In addition of increasing the amount of PHA in plants , expression of the thioesterase leads to a predictable change in the composition of the PHA, i.e. since the *C. lanceolata* FatB3 thioesterase has the highest affinity for saturated C10 fatty acyl-ACP, there is a corresponding large increase in hydroxydecanoic acid (H10) present in the plant PHA (Table 8). Thus, expression of fatty acid modifying enzymes in conjunction with a PHA synthase in plants not only leads to an increase in the amount of PHA synthesized in plants, but also leads to a predictable changes in the PHA monomer composition, e.g. co-expression of a short-chain fatty acyl-ACP thioesterase would lead to an increase in the proportion of short-chain hydroxyacid monomers in plant PHA, co-expression of a long-chain fatty acyl-ACP thioesterase would lead to an increase in the proportion of long-chain hydroxyacid monomers in plant PHA, co-expression of a fatty acyl hydroxylase would lead to an increase in the proportion of hydroxylated hydroxyacid monomers in plant PHA, co-expression of a fatty acyl epoxidase would lead to an increase in the proportion of epoxidated monomers in plant PHA, co-expression of a fatty acyl acetylase would lead to an increase in the proportion of acetylated hydroxyacid monomers in plant PHA, and co-expression of a fatty acyl desaturase would lead to an increase in the

proportion of unsaturated hydroxyacid monomers in plant PHA. Increase in flux of lipids through the β-oxidation cycle could also be accomplished by overexpressing the key regulators (i.e. transcriptional factors) involved in the up-regulation of the entire β-oxidation cycle pathway during germination or senescence. This last approach would have the advantage of turning-on the β-oxidation cycle in tissues which normally have only low activity, such as the developing seeds of oil crops.

The examples herein point out the impact of fatty acid modifying enzymes for the production of novel PHA in transgenic plants expressing a PHA synthase. One key enzyme appears to be a 3-hydroxy-acyl-CoA epimerase. Although the normal function of the epimerase is to convert D-3-hydroxy-acyl-CoAs to the L-form required for the action of the L-3-hydroxy-acyl-CoA dehydrogenase, the reverse reaction of the epimerase can be responsible for converting the L-form to the D-form, which is essential for the activity of the PHA synthase. For that purpose the epimerase is important for the supply of the substrates for the PHA synthase derived from β-oxidation in the peroxisomes. Recombinant forms of such an epimerase activity expressed in peroxisomes or in other plant cell compartments like the cytoplasm or the plastids could play an important role in the production of PHA in transgenic plants. It is possible that the slow rate of the epimerase "reverse reaction" could be the major factor limiting the supply of substrates for the PHA synthase. The substrate limitation due to this could be the reason why PHA synthesis seemed to have reached a maximum in seedlings germinated both in the light and in the dark in liquid medium supplemented with TWEEN-20, which contains only saturated fatty acids.

The importance of certain fatty acid desaturases is highlighted by Table 3, wherein petroselinic acid (C18:1, 6-cis) was supplied to germinating PHAC1#3.3 seedlings in liquid medium, resulting in the specific increase of the H14 monomer. This indicated that any fatty acid containing unsaturated bonds starting at even-numbered carbons directly gives rise to the appropriate D-3-hydroxy-acyl-CoAs during β-oxidation, thus bypassing the otherwise necessary "reverse reaction" of the epimerase to generate the D-intermediates. Similarly the H8 and the H8:1 monomer are predicted to originate from the unsaturated fatty

acids linoleic acid (C18:2, 9,12-all cis) and linolenic acid (C18:3, 9,12,15-all cis). For that reason any plant containing high levels of fatty acids with unsaturated bonds starting at even-numbered carbons could be of interest for the production of PHA_{mcl}, or the transgenic expression of suitable fatty acid desaturases producing such unsaturated fatty acids in plants containing the PHA synthase would be similarly attractive for PHA production and monomer manipulation.

The examples herein demonstrate that a peroxisomally-located PHA synthase is able to divert intermediates from β-oxidation for their incorporation into PHA. The existence of the required D-3-hydroxy-acyl-CoA substrates was important for the synthesis of PHA. In light of the present disclosure, one may predict that PHA can be produced in a similar manner in any other compartment of any plant cell, provided that a supply of such D-3-hydroxy-acyl-CoA intermediates is present due either to an endogenous metabolic pathway or due to an artificially created pathway utilizing expression of transgenes. Fatty acid biosynthesis occurs in the plastids in plant cells, and modifications of this pathway could turn the plastids into a suitable source of D-3-hydroxy-acyl-CoA intermediates, which could subsequently be used to produce PHA either in the plastid itself or in other cell compartments.

EXAMPLE 17: Protein analysis

Leaves from transgenic plants were homogenized in 200 mM Tris-HCl (pH 7.5), 250 mM EDTA, 5 mM dithiothreitol and 1 mM phenylmethylsulfonyl fluoride. The homogenate was clarified by centrifugation and protein analyzed by Western blot using the ECL detection system (Amersham, Arlington Heights, IL).

EXAMPLE 18: Immunolocalization

Transgenic plants were grown on media containing MS salts, 1% sucrose, 0.7% agar and 50 μg/mL kanamycin for either 7 days in the light or 1 day in the light followed by 6 days in the dark. Whole plants were fixed for 2 hours at room temperature in 4%

formaldehyde, 0.5% glutaraldehyde, 50 mM sodium cacodylate pH 7.3. The tissue samples were dehydrated in an ethanol series and embedded in LR White resin. Ultra thin sections were cut using a microtome, mounted on formvar-coated gold grids and blocked in 0.8% (w/v) bovine serum albumin, 0.1% (w/v) gelatine, 5% (w/v) normal goat serum and 2 mM sodium azide in PBS (10 mM sodium phosphate, 150 mM sodium chloride, pH 7.4). Grids were incubated for 1 hour at room temperature with antiserum against PHA synthase (1:50), glycolate oxidase (1:2000) and isocitrate lyase (1:1000) in the blocking solution followed by a 4 hour incubation at room temperature with a 1:50 dilution of gold-conjugated goat anti-rabbit antibodies (15 nm gold particles) in PBS. Immunolabeled sections were doubled-stained with uranyl acetate and lead citrate and viewed with a Jeol JEM transmission electron microscope.

EXAMPLE 19: PHA extraction and analysis

Fresh or dried frozen plant material was ground in a mortar and lyophilized. The powder was extracted with methanol in a Soxhlet apparatus for 24 hours followed by PHA extraction with chloroform for 24 hours, both at 85°C. The PHA-containing chloroform was concentrated under reduced pressure and extracted once with water to remove residual solid particles. PHA was precipitated by the addition of 10 volumes of cold methanol and subsequently washed by two cycles of chloroform solubilisation and methanol precipitation. PHA dissolved in chloroform was transesterified by acid methanolysis (Huijberts, G. N. et al., *Appl. Environ. Microbiol.* 58: 536-544 (1992)) and analyzed by gas-chromatography and mass spectrometry (GC-MS) using a Hewlett-Packard 5890 gas chromatograph (30 m long HP-5MS column) coupled to a Hewlett-Packard 5972 mass spectrometer (Hewlett Packard, Palo Alto, CA). Molecular weight determination of PHA samples were determined by gel permeation chromatography on a Waters 150 CV (Waters Corp., Milford, MA) equipped with a differential refractive index detector and an on-line viscometer and three ultrastyragel columns in series (10^4, 10^5 and 10^6 Å). Samples were prepared in dichloromethane and calibration performed using polystyrene standards.

EXAMPLE 20: Plant Vectors

In plants, transformation vectors capable of introducing encoding DNAs involved in PHA biosynthesis are easily designed, and generally contain one or more DNA coding sequences of interest under the transcriptional control of 5' and 3' regulatory sequences. Such vectors generally comprise, operatively linked in sequence in the 5' to 3' direction, a promoter sequence that directs the transcription of a downstream heterologous structural DNA in a plant; optionally, a 5' non-translated leader sequence; a nucleotide sequence that encodes a protein of interest; and a 3' non-translated region that encodes a polyadenylation signal which functions in plant cells to cause the termination of transcription and the addition of polyadenylate nucleotides to the 3' end of the mRNA encoding said protein. Plant transformation vectors also generally contain a selectable marker. Typical 5'-3' regulatory sequences include a transcription initiation start site, a ribosome binding site, an RNA processing signal, a transcription termination site, and/or a polyadenylation signal. Vectors for plant transformation have been reviewed in Rodriguez et al. (Vectors: A Survey of Molecular Cloning Vectors and Their Uses, Butterworths, Boston. (1988)), Glick et al. (Methods in Plant Molecular Biology and Biotechnology, CRC Press, Boca Raton, Fla. (1993)), and Croy (Plant Molecular Biology Labfax, Hames and Rickwood (Eds.), BIOS Scientific Publishers Limited, Oxford, UK. (1993)).

EXAMPLE 21: Plant Promoters

Plant promoter sequences can be constitutive or inducible, environmentally- or developmentally-regulated, or cell- or tissue-specific. Often-used constitutive promoters include the CaMV 35S promoter (Odell et al., *Nature* 313: 810 (1985)), the enhanced CaMV 35S promoter, the Figwort Mosaic Virus (FMV) promoter (Richins et al., *Nucleic Acids Res.* 20: 8451 (1987)), the mannopine synthase (*mas*) promoter, the nopaline synthase (*nos*) promoter, and the octopine synthase (*ocs*) promoter. Useful inducible promoters include promoters induced by salicylic acid or polyacrylic acids (PR-1, Williams , S. W. et al, *Biotechnology* 10: 540-543 (1992)), induced by application of safeners (substituted benzenesulfonamide herbicides, Hershey, H.P. and Stoner, T.D., *Plant Mol. Biol.* 17: 679-

690 (1991)), heat-shock promoters (Ou-Lee et al., *Proc. Natl. Acad. Sci U.S.A.* 83: 6815 (1986); Ainley et al., *Plant Mol. Biol.* 14: 949 (1990)), a nitrate-inducible promoter derived from the spinach nitrite reductase gene (Back et al., *Plant Mol. Biol.* 17: 9 (1991)), hormone-inducible promoters (Yamaguchi-Shinozaki et al., *Plant Mol. Biol.* 15: 905 (1990); Kares et al., *Plant Mol. Biol.* 15: 905 (1990)), and light-inducible promoters associated with the small subunit of RuBP carboxylase and LHCP gene families (Kuhlemeier et al., *Plant Cell* 1: 471 (1989); Feinbaum et al., *Mol. Gen. Genet.* 226: 449 (1991); Weisshaar et al., *EMBO J.* 10: 1777 (1991); Lam and Chua, *J. Biol. Chem.* 266: 17131 (1990); Castresana et al., *EMBO J.* 7: 1929 (1988); Schulze-Lefert et al., *EMBO J.* 8: 651 (1989)). Examples of useful tissue-specific, developmentally-regulated promoters include the β-conglycinin 7S promoter (Doyle et al., *J. Biol. Chem.* 261: 9228 (1986); Slighton and Beachy, *Planta* 172: 356 (1987)), and seed-specific promoters (Knutzon et al., *Proc. Natl. Acad. Sci U.S.A.* 89: 2624 (1992); Bustos et al., *EMBO J.* 10: 1469 (1991); Lam and Chua, *Science* 248: 471 (1991); Stayton et al., *Aust. J. Plant. Physiol.* 18: 507 (1991)). Plant functional promoters useful for preferential expression in seed plastids include those from plant storage protein genes and from genes involved in fatty acid biosynthesis in oilseeds. Examples of such promoters include the 5' regulatory regions from such genes as napin (Kridl et al., *Seed Sci. Res.* 1: 209 (1991)), phaseolin, zein, soybean trypsin inhibitor, ACP, stearoyl-ACP desaturase, and oleosin. Seed-specific gene regulation is discussed in EP 0 255 378. Promoter hybrids can also be constructed to enhance transcriptional activity (Comai, L. and Moran, P.M., U.S. Patent No. 5,106,739, issued April 21, 1992), or to combine desired transcriptional activity and tissue specificity.

EXAMPLE 22: Plant transformation and regeneration

A variety of different methods can be employed to introduce such vectors into plant protoplasts, cells, callus tissue, leaf discs, meristems, etcetera, to generate transgenic plants, including *Agrobacterium*-mediated transformation, particle gun delivery, microinjection, electroporation, polyethylene glycolmediated protoplast transformation, liposome-mediated transformation, etc. (reviewed in Potrykus, *Ann. Rev. Plant Physiol. Plant Mol. Biol.* 42: 205 (1991)). In general, transgenic plants comprising cells containing and expressing

DNAs encoding enzymes facilitating PHA biosynthesis can be produced by transforming plant cells with a DNA construct as described above via any of the foregoing methods; selecting plant cells that have been transformed on a selective medium; regenerating plant cells that have been transformed to produce differentiated plants; and selecting a transformed plant which expresses the enzyme-encoding nucleotide sequence.

Specific methods for transforming a wide variety of dicots and obtaining transgenic plants are well documented in the literature (Gasser and Fraley, *Science* 244: 1293 (1989); Fisk and Dandekar, *Scientia Horticulturae* 55: 5 (1993); Christou, *Agro Food Industry Hi Tech*, p.17 (1994); and the references cited therein).

Successful transformation and plant regeneration have been reported in the monocots as follows: asparagus (*Asparagus officinalis*; Bytebier et al., *Proc. Natl. Acad. Sci. U.S.A.* 84: 5345 (1987)); barley (*Hordeum vulgarae*; Wan and Lemaux, *Plant Physiol.* 104: 37 (1994)); maize (*Zea mays*; Rhodes et al., Science 240: 204 (1988); Gordon-Kamm et al., *Plant Cell* 2: 603 (1990); Fromm et al., *Bio/Technology* 8: 833 (1990); Koziel et al., *Bio/Technology* 11: 194 (1993)); oats (*Avena sativa*; Somers et al., *Bio/Technology* 10: 1589 (1992)); orchardgrass (*Dactylis glomerata*; Horn et al., *Plant Cell Rep.* 7: 469 (1988)); rice (*Oryza sativa*, including indica and japonica varieties; Toriyama et al., *Bio/Technology* 6: 10 (1988); Zhang et al., *Plant Cell Rep.* 7: 379 (1988); Luo and Wu, *Plant Mol. Biol.* Rep. 6: 165 (1988); Zhang and Wu, *Theor. Appl. Genet.* 76: 835 (1988); Christou et al., *Bio/Technology* 9: 957 (1991)); rye (*Secale cereale*; De la Pena et al., *Nature* 325: 274 (1987)); sorghum (*Sorghum bicolor*; Cassas et al., *Proc. Natl. Acad. Sci. USA* 90: 11212 (1993)); sugar cane (*Saccharum* spp.; Bower and Birch, *Plant J.* 2: 409 (1992)); tall fescue (*Festuca arundinacea*; Wang et al., *Bio/Technology* 10: 691 (1992)); turfgrass (*Agrostis palustris*; Zhong et al., *Plant Cell Rep.* 13: 1 (1993)); wheat (*Triticum aestivum*; Vasil et al., *Bio/Technology* 10: 667 (1992); Weeks et al., *Plant Physiol.* 102: 1077 (1993); Becker et al., *Plant J.* 5: 299 (1994)), and alfalfa (Masoud, S.A. et al., *Transgen. Res.* 5: 313 (1996)).

EXAMPLE 23: Host plants

Particularly useful plants for PHA production include those that produce carbon substrates which can be employed for PHA biosynthesis, including tobacco, wheat, potato, *Arabidopsis*, and high oil seed plants such as corn, soybean, canola, oil seed rape, sunflower, flax, peanut, sugarcane, switchgrass, and alfalfa.

If the host plant of choice does not produce the requisite fatty acid substrates in sufficient quantities, it can be modified, for example by mutagenesis or genetic transformation, to block or modulate the glycerol ester and fatty acid biosynthesis or degradation pathways so that it accumulates the appropriate substrates for PHA production. Expression of enzymes such as acyl-ACP thioesterase, fatty acyl hydroxylase, and yeast multifunctional protein (MFP) may serve to increase the flux of substrates in the peroxisome, leading to higher levels of PHA biosynthesis.

EXAMPLE 24: Nucleic acid mutation and hybridization

Variations in the nucleic acid sequence encoding a fusion protein may lead to mutant protein sequences that display equivalent or superior enzymatic characteristics when compared to the sequences disclosed herein. This invention accordingly encompasses nucleic acid sequences which are similar to the sequences disclosed herein, protein sequences which are similar to the sequences disclosed herein, and the nucleic acid sequences that encode them. Mutations may include deletions, insertions, truncations, substitutions, fusions, and the like.

Mutations to a nucleic acid sequence may be introduced in either a specific or random manner, both of which are well known to those of skill in the art of molecular biology. A myriad of site-directed mutagenesis techniques exist, typically using oligonucleotides to introduce mutations at specific locations in a nucleic acid sequence. Examples include single strand rescue (Kunkel, T. *Proc. Natl. Acad. Sci. U.S.A.*, 82: 488-492 (1985)), unique site elimination (Deng and Nickloff, *Anal. Biochem.* 200: 81 (1992)),

nick protection (Vandeyar, et al. *Gene* 65: 129-133 (1988)), and PCR (Costa, et al. *Methods Mol. Biol.* 57: 31-44 (1996)). Random or non-specific mutations may be generated by chemical agents (for a general review, see Singer and Kusmierek, *Ann. Rev. Biochem.* 52: 655-693 (1982)) such as nitrosoguanidine (Cerda-Olmedo et al., *J. Mol. Biol.* 33:705-719 (1968); Guerola, et al. *Nature New Biol.* 230: 122-125 (1971)) and 2-aminopurine (Rogan and Bessman, *J. Bacteriol.* 103: 622-633 (1970)), or by biological methods such as passage through mutator strains (Greener et al. *Mol. Biotechnol.* 7: 189-195 (1997)).

Nucleic acid hybridization is a technique well known to those of skill in the art of DNA manipulation. The hybridization properties of a given pair of nucleic acids is an indication of their similarity or identity. Mutated nucleic acid sequences may be selected for their similarity to the disclosed nucleic acid sequences on the basis of their hybridization to the disclosed sequences. Low stringency conditions may be used to select sequences with multiple mutations. One may wish to employ conditions such as about 0.15 M to about 0.9 M sodium chloride, at temperatures ranging from about 20°C to about 55°C. High stringency conditions may be used to select for nucleic acid sequences with higher degrees of identity to the disclosed sequences. Conditions employed may include about 0.02 M to about 0.15 M sodium chloride, about 0.5% to about 5% casein, about 0.02% SDS and/or about 0.1% N-laurylsarcosine, about 0.001 M to about 0.03 M sodium citrate, at temperatures between about 50°C and about 70°C. More preferably, high stringency conditions are 0.02 M sodium chloride, 0.5% casein, 0.02% SDS, 0.001 M sodium citrate, at a temperature of 50°C.

EXAMPLE 25: Determination of homologous and degenerate nucleic acid sequences

Modification and changes may be made in the sequence of the proteins of the present invention and the nucleic acid segments which encode them and still obtain a functional molecule that encodes a protein with desirable properties. The following is a discussion based upon changing the amino acid sequence of a protein to create an equivalent, or possibly an improved, second-generation molecule. The amino acid changes

may be achieved by changing the codons of the nucleic acid sequence, according to the codons given in Table 11.

Table 11: Codon degeneracies of amino acids

Amino acid	One letter	Three letter	Codons
Alanine	A	Ala	GCA GCC GCG GCT
Cysteine	C	Cys	TGC TGT
Aspartic acid	D	Asp	GAC GAT
Glutamic acid	E	Glu	GAA GAG
Phenylalanine	F	Phe	TTC TTT
Glycine	G	Gly	GGA GGC GGG GGT
Histidine	H	His	CAC CAT
Isoleucine	I	Ile	ATA ATC ATT
Lysine	K	Lys	AAA AAG
Leucine	L	Leu	TTA TTG CTA CTC CTG CTT
Methionine	M	Met	ATG
Asparagine	N	Asn	AAC AAT
Proline	P	Pro	CCA CCC CCG CCT
Glutamine	Q	Gln	CAA CAG
Arginine	R	Arg	AGA AGG CGA CGC CGG CGT
Serine	S	Ser	AGC AGT TCA TCC TCG TCT
Threonine	T	Thr	ACA ACC ACG ACT
Valine	V	Val	GTA GTC GTG GTT
Tryptophan	W	Trp	TGG
Tyrosine	Y	Tyr	TAC TAT

Certain amino acids may be substituted for other amino acids in a protein sequence without appreciable loss of enzymatic activity. It is thus contemplated that various changes may be made in the peptide sequences of the disclosed protein sequences, or their corresponding nucleic acid sequences without appreciable loss of the biological activity.

In making such changes, the hydropathic index of amino acids may be considered. The importance of the hydropathic amino acid index in conferring interactive biological function on a protein is generally understood in the art (Kyte and Doolittle, *J. Mol. Biol.*, 157: 105-132 (1982)). It is accepted that the relative hydropathic character of the amino acid contributes to the secondary structure of the resultant protein, which in turn defines the

interaction of the protein with other molecules, for example, enzymes, substrates, receptors, DNA, antibodies, antigens, and the like.

Each amino acid has been assigned a hydropathic index on the basis of their hydrophobicity and charge characteristics. These are: isoleucine (+4.5); valine (+4.2); leucine (+3.8); phenylalanine (+2.8); cysteine/cystine (+2.5); methionine (+1.9); alanine (+1.8); glycine (-0.4); threonine (-0.7); serine (-0.8); tryptophan (-0.9); tyrosine (-1.3); proline (-1.6); histidine (-3.2); glutamate/glutamine/aspartate/asparagine (-3.5); lysine (-3.9); and arginine (-4.5).

It is known in the art that certain amino acids may be substituted by other amino acids having a similar hydropathic index or score and still result in a protein with similar biological activity, i.e., still obtain a biologically functional protein. In making such changes, the substitution of amino acids whose hydropathic indices are within ±2 is preferred, those within ±1 are more preferred, and those within ±0.5 are most preferred.

It is also understood in the art that the substitution of like amino acids may be made effectively on the basis of hydrophilicity. U.S. Patent No. 4,554,101 (Hopp, T.P., issued November 19, 1985) states that the greatest local average hydrophilicity of a protein, as governed by the hydrophilicity of its adjacent amino acids, correlates with a biological property of the protein. The following hydrophilicity values have been assigned to amino acids: arginine/lysine (+3.0); aspartate/glutamate (+3.0 ±1); serine (+0.3); asparagine/glutamine (+0.2); glycine (0); threonine (-0.4); proline (-0.5 ±1); alanine/histidine (-0.5); cysteine (-1.0); methionine (-1.3); valine (-1.5); leucine/isoleucine (-1.8); tyrosine (-2.3); phenylalanine (-2.5); and tryptophan (-3.4).

It is understood that an amino acid may be substituted by another amino acid having a similar hydrophilicity score and still result in a protein with similar biological activity, i.e., still obtain a biologically functional protein. In making such changes, the substitution of amino acids whose hydropathic indices are within ±2 is preferred, those within ±1 are more preferred, and those within ±0.5 are most preferred.

As outlined above, amino acid substitutions are therefore based on the relative similarity of the amino acid side-chain substituents, for example, their hydrophobicity, hydrophilicity, charge, size, and the like. Exemplary substitutions which take various of the foregoing characteristics into consideration are well known to those of skill in the art and include: arginine and lysine; glutamate and aspartate; serine and threonine; glutamine and asparagine; and valine, leucine, and isoleucine. Changes which are not expected to be advantageous may also be used if these resulted in functional fusion proteins.

All of the compositions and/or methods disclosed and claimed herein can be made and executed without undue experimentation in light of the present disclosure. While the compositions and methods of this invention have been described in terms of preferred embodiments, it will be apparent to those of skill in the art that variations may be applied to the compositions and/or methods and in the steps or in the sequence of steps of the methods described herein without departing from the concept, spirit and scope of the invention. More specifically, it will be apparent that certain agents which are both chemically and physiologically related may be substituted for the agents described herein while the same or similar results would be achieved. All such similar substitutes and modifications apparent to those skilled in the art are deemed to be within the spirit, scope and concept of the invention.